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CHARACTERIZATION OF NEONATAL PORCINE ISLETS AS AN ALTERNATIVE SOURCE OF INSULIN-PRODUCING TISSUE FOR TRANSPLANTATION INTO PATIENTS WITH INSULIN-DEPENDENT DIABETES MELLITUS

by

Gina Ranile Rayat ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Surgery

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHARACTERIZATION OF NEONATAL PORCINE ISLETS AS AN ALTERNATIVE SOURCE OF INSULIN-PRODUCING TISSUE FOR TRANSPLANTATION INTO PATIENTS WITH INSULIN-DEPENDENT DIABETES MELLITUS in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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This text is dedicated to Stanley Ross Banks and to my *Nanay* and *Tatay* who have given their unending love and support all through these years.

ABSTRACT

Neonatal porcine islets (NPI) may potentially solve the shortage of tissues for human transplantation, however, it is critical that we must further characterized these tissues before they can be used in clinical transplantation. It is not known to what extent NPI will be susceptible to destruction by both humoral and cell-mediated mechanisms. Galα(1,3)Gal is the major xenoantigen responsible for hyperacute rejection of pig organs. This antigen is recognized by human xenoreactive natural antibodies and when these bind and activate complement, the transplanted organs or tissues are rapidly destroyed. It is generally believed that Galα(1,3)Gal is not expressed on islet endocrine cells and therefore it has been speculated that these cells would be spared from humoral-mediated destruction. However, we found that a subset of NPI consists of cells which expressed Galα(1,3)Gal and expression was detected on both endocrine and non-endocrine cells. Interestingly, both Gal-positive and Gal-negative cells were susceptible to humoral-mediated destruction *in vitro*. To further characterize the expression of this antigen on porcine islets, 9-day cultured (non-matured) NPI, NPI which have been matured by *in vitro* culture, or by transplantation into nude mice, and adult pig islets were immunostained with IB4 lectin and/or antibodies to insulin and cytokeratin 7. Galα(1,3)Gal expression on porcine islet cells is age-dependent being most abundant on non-matured β cells. This may explain in part why other studies did not detect this antigen on endocrine cells.

One strategy to prevent destruction of islets by human immune cells is to use immunoisolation device such as microcapsules. To test the efficacy of

microencapsulation in protecting NPI from the cytolytic effects of human antibody and complement we exposed microencapsulated NPI to human serum and complement. Microencapsulation protected NPI from the *in vitro* cytolytic effects of human antibody and complement and the microencapsulated tissues can reverse diabetes in nude mice. Finally, to examine the susceptibility of NPI to cell-mediated immune injury, the proliferative response of human peripheral blood lymphocytes, as well as the ability of natural killer (NK) and cytotoxic T lymphocytes (CTL) to lyse NPI cells were measured. NPI cells were not susceptible to injury mediated by NK cells and/or CTL *in vitro*.

Taken together, these studies provided important information on the nature of human immunological responses against NPI and the potential of these tissues to one day be successfully transplanted into patients with type 1 diabetes.

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TABLE OF CONTENTS

CHAPTER I

GENERAL INTRODUCTION 1

I-A) DIABETES MELLITUS 1
- 1. Insulin-Dependent Diabetes Mellitus 2
- 2. Etiology of IDDM 3
  - a) Genetic factors 3
  - b) Environmental factors 4
    - i) viruses 4
    - ii) dietary factors 5

I-B) CURRENT TREATMENTS FOR IDDM 7
- 1. Subcutaneous Insulin Injection 7
- 2. Pancreas Transplantation 8
- 3. Islet Transplantation 10

I-C) BARRIERS TO SUCCESS OF ISLET TRANSPLANTATION 16
- 1. Limited Supply of Human Islet Tissue 16
  - a) Adult porcine islets 18
  - b) Fetal porcine islets 19
  - c) Neonatal porcine islets 20
- 2. Immunological Responses to Neonatal Porcine Islets 22
  - a) Humoral-mediated immune response 22
    - i) hyperacute rejection 22
    - ii) Galα(1,3)Gal antigen 25
  - b) T cell-mediated immune response 29

I-D) STRATEGIES FOR PREVENTING REJECTION OF NEONATAL PORCINE ISLETS 31
- 1. Humoral-Mediated Rejection 31
- 2. Cell-Mediated Rejection 32

I-E) PANCREATIC B CELL GROWTH, DEVELOPMENT, AND REGENERATION 38

I-F) OBJECTIVES AND GENERAL OUTLINE 42

I-G) REFERENCES 45

CHAPTER II
EXPRESSION OF GALα(1,3)GAL ON NEONATAL PORCINE ISLET BETA CELLS AND SUSCEPTIBILITY TO HUMAN ANTIBODY-COMPLEMENT LYSIS 73

II-A) INTRODUCTION 73

II-B) MATERIALS AND METHODS 75
- 1. Preparation of Neonatal Porcine Islets 75
- 2. Immunohistochemical Analysis 75
- 3. Characterization of Dissociated NPI Cells 76
- 4. Cytotoxicity Assay and Morphology 77
- 5. Statistical Analysis 79

II-C) RESULTS 80
- 1. Expression of Galα(1,3)Gal on Neonatal Porcine Pancreas and Islet Cells 80
- 2. Characterization of Neonatal Porcine Islet Cells 82
- 3. Susceptibility to Antibody/Complement-Mediated Lysis 86

II-D) DISCUSSION 88

II-E) REFERENCES 93

CHAPTER III
GALα(1,3)GAL EXPRESSION ON PORCINE ISLET CELLS IS AGE-DEPENDENT 96

III-A) INTRODUCTION 96

III-B) MATERIALS AND METHODS 98
- 1. Preparation of Neonatal Porcine and Adult Pig Islets 98
- 2. *In Vitro* Maturation of NPI 99
- 3. Preparation of NPI and Adult Pig Islet Cells 99
- 4. *In Vivo* Maturation of NPI 100
- 5. Immunocyto/histochemical Analysis 100
  - a) Single label immunocytochemical analysis 100
  - b) Double label immunohistochemical analysis 102
- 6. Fluorescence Activated Cell Sorter (FACS) Analysis 102
- 7. Statistical Analysis 103

III-C) RESULTS 103
- 1. Cellular Composition of Non-Mature, *In Vitro* Mature NPIs, and Adult Pig Islets 103
- 2. Distribution of CK7 and Insulin in Galα(1,3)Gal-Positive and -Negative NPI Cells 104

3. *In Vivo* Expression of Galα(1,3)Gal and CK7 104

III-D) DISCUSSION 109

III-E) REFERENCES 113

CHAPTER IV
MICROENCAPSULATION OF NEONATAL PORCINE ISLETS: PROTECTION FROM HUMAN ANTIBODY/COMPLEMENT-MEDIATED CYTOLYSIS *IN VITRO* AND LONG-TERM REVERSAL OF DIABETES IN NUDE MICE 116

IV-A) INTRODUCTION 116

IV-B) MATERIALS AND METHODS 117
1. Animals 117
2. Preparation and Encapsulation of NPI 118
3. *In Vitro* Human Antibody/Complement-Mediated Cytotoxicity Assay 119
4. Transplantation of Encapsulated NPI and Metabolic Follow-up 120
5. Morphological assessment of non-encapsulated and encapsulated NPI pre-and post-transplantation 121
6. Statistical Analysis 122

IV-C) RESULTS 122
1. Cytotoxicity of Human Serum and Complement 122
2. Transplantation of NPI Into Diabetic Nude Mice 125

IV-D) DISCUSSION 129

IV-E) REFERENCES 133

CHAPTER V
NEONATAL PORCINE ISLET CELLS INDUCE HUMAN CD4+ BUT NOT CD8+ LYMPHOCYTE PROLIFERATION AND RESIST CELL-MEDIATED CYTOLYTIC INJURY *IN VITRO* 135

V-A) INTRODUCTION 135

V-B) MATERIALS AND METHODS 137
1. Preparation of NPI Cells 137
2. Isolation of Human PBL 138
3. PBL Proliferation Assay 139
4. Cytotoxicity Assay 139
5. Indirect Immunofluorescence and Immunocytochemical Analyses 141
6. Statistical Analysis 142

V-C) RESULTS 142
1. Recognition of NPI Cell Xenoantigen by Human Lymphocytes 142
2. Human NK and CTL-Mediated Lysis of NPI Cells 143
3. Expression of SLA-I and SLA-II on NPI Cells 146
4. The Effect of Pro-Inflammatory Cytokine Pre-Treatment of NPI Cells on Anti-NPI Cells Activity 148
5. Determination of the Effector Mechanism used by CTL to Lyse Neonatal Porcine Splenocytes 150

V-D) DISCUSSION 151

V-E) REFERENCES 156

CHAPTER VI
GENERAL DISCUSSION AND CONCLUSION 161

VI-A) GENERAL DISCUSSION 161

VI-B) CONCLUSION 167

VI-C) REFERENCES 169

APPENDIX
DETERMINATION OF XENOREACTIVE ANTIBODIES IN SERUM OF YOUNG RABBIT 174

A-1) OBJECTIVE 174

A-2) RATIONALE 174

A-3) EXPERIMENTAL PROTOCOL 175

A-4) RESULTS 175

A-5) CONCLUSION 177

A-6) REFERENCES 178

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1-1	Comparison between whole pancreas and islet transplantation	11
1-2	Minimal requirements to be met by islet preparations intended for clinical islet transplantation	16
2-1	Cellular composition of neonatal porcine islet cells.	83
2-2	Susceptibility of neonatal porcine islet cells to human antibody/complement-mediated lysis.	87
3-1	Cellular composition of non-mature, *in vitro* mature, NPI, and adult pig islets.	106
3-2	Expression of CK7 and/or insulin in Galα(1,3)Gal-positive and -negative non-mature NPI cells.	106
4-1	Effect of human serum and complement on non-encapsulated and encapsulated NPI.	123
4-2	Effect of human serum and complement on the insulin secretory capacity of NPI.	124
4-3	Metabolic follow-up of diabetic nude mice transplanted with 2,000 NPI.	127
5-1	Expression of SLA-I and SLA-II on NPI cells and neonatal porcine splenocytes.	146
5-2	Cellular composition of SLA-I-positive and -negative NPI cells.	148

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
1-1	Blood glucose levels of diabetic nude mice transplanted with 2,000 NPI.	22
1-2	Reciprocal evolution of Galα(1,3)Gal epitopes and the anti-Gal antibodies in mammals.	26
1-3	Schematic diagram of microencapsulated islet.	38
2-1	Light micrographs of native neonatal porcine pancreas, freshly digested neonatal porcine pancreatic aggregates, 9-day culture islets, and 9-day culture dissociated islet cells.	81
2-2	Light micrograph of NPI cells stained with FITC-conjugated IB4 lectin.	83
2-3	FACS analysis of PAEC and dissociated 9-day culture NPI stained with FITC-conjugated IB4 lectin.	84
2-4	Binding of human xenoreactive antibodies to NPI cells.	85
2-5	Electron micrographs of dissociated NPI cells exposed to media, HI-pooled human AB serum containing no complement activity, HI-pooled human Ab serum with rabbit complement or with complement only.	87
3-1	Expression of Galα(1,3)Gal and insulin on NPI xenografts	107
3-2	Expression of CK7 and insulin on NPI xenografts	108
4-1	Blood glucose values in alloxan-induced diabetic nude mice transplanted with NPI.	126
4-2	Morphology of encapsulated NPI before and after implantation to alloxan-induced diabetic nude mice.	128
5-1	Proliferation of unstimulated human PBL, or PBL stimulated by co-culture with neonatal porcine splenocytes, or NPI cells.	144

5-2 Human CD4+ and CD8+ T cells proliferation in response to NPI cells stimulation. 144

5-3 Human NK cell activity against NPI cells or neonatal porcine splenocytes. 145

5-4 Human CTL lyse neonatal porcine splenocytes but not NPI cells. 145

5-5 SLA-I and SLA-II expression on NPI cells and neoanatal porcine splenocytes. 147

5-6 Cell-mediated lysis of NPI cells after treatment with cytokine-rich supernatant. 149

5-7 Lysis of NPI cells and neonatal porcine splenocytes is enhanced by co-culture in the presence of PHA. 149

5-8 Lysis of neonatal porcine splenocytes was inhibited completely by either pre-treatment of the PBL with V-ATPase inhibitor concanamycin A or chelation of free calcium. 151

A-1 Binding of rabbit anti-porcine antibodies on NPI cells treated with adult rabbit serum, young rabbit serum, secondary antibody as control. 176

LIST OF ABBREVIATIONS

α1,3GT	α1,3 galactosyltransferase
ABC	avidin-biotin complex
ADCC	antibody-dependent cell-mediated cytotoxicity
ALG	anti-lymphocyte globulin
ALS	anti-lymphocyte serum
AP	alkaline phosphatase
APC	antigen-presenting cells
Apo J	apoprotein J
AVR	acute vascular rejection
BSA	bovine serum albumin
BS-1	*Bandeiraea simplicifolia*-1
CH_{50}	50% complement hemolytic activity
CK	cytokeratins
CTL	cytotoxic T lymphocytes
DAB	3,3-diaminobenzidinetetrahydrochloride
DAF	decay accelerating factor
DCCT	Diabetes Control and Complications Trial
dpc	days postcoitum
DXR	delayed xenograft rejection
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FasL	Fas ligand

FasR	Fas receptor
FITC	fluorescein isothiocyanate
GAD	glutamic acid decarboxylase
Galα(1,3)Gal	galactoseα(1,3)galactose
HAR	hyperacute rejection
HBSS	Hank's balanced salt solution
HI	heat-inactivated
HLA	human leukocyte antigens
HP	horseradish peroxidase
IAA	insulin autoantibodies
IB4	isolectin-B4
ICA	islet cell antibodies
IDDM	insulin-dependent diabetes mellitus
IDX-1	islet/duodenum homeobox-1
IF	intermediate filaments
IL1-α	interleukin 1-α
IFN-γ	interferon-γ
i.p.	intraperitoneal
IPF-1	insulin promoter factor-1
IPGTT	intraperitoneal glucose tolerance test
ISL-1	islet-1
IUF-1	insulin upstream factor-1
k.c.	kidney capsule

MCP	membrane cofactor protein
MHC	major histocompatibility complex
MODY	maturity-onset diabetes mellitus of the young
MRDM	malnutrition-related diabetes mellitus
NIDDM	non-insulin-dependent diabetes mellitus
NK	natural killer
NOD	non-obese diabetic
NPI	neonatal porcine islets
OGTT	oral glucose tolerance test
PAEC	porcine aortic endothelial cells
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDX-1	pancreatic and duodenal homeobox gene-1
PERV	porcine endogenous retrovirus
PHA	phytohemagglutinin
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SGP-2	sulfated glycoprotein-2
SLA	swine leukocyte antigens
SP-40-40	soluble protein-40-40
STF-1	somatostatin transactivating factor-1
TGF-β	transforming growth factor-β

TNF-α	tumor necrosis factor-α
UW	University of Wisconsin
WHO	World Health Organization
XNA	xenoreactive natural antibodies

CHAPTER I

GENERAL INTRODUCTION

I-A) DIABETES MELLITUS

Diabetes is a Greek word which means a fountain and **mellitus** is the Latin word for honey thus, **diabetes mellitus** literally means a "fountain of honey" (1). In clinical terms, diabetes mellitus is a chronic disorder in which the pancreas is unable to produce insulin, does not produce enough insulin to meet the body's needs or the body cannot use the insulin that is produced. As a result high levels of glucose in the blood and urine are observed. According to the Diabetes Resources Network in Canada (2), diabetes mellitus is the leading cause of death by disease in Canada and also the leading cause of new blindness in adults. It is responsible for 25% of cardiac surgery, 40% of end-stage renal disease, and 50% of all non-traumatic amputations. The percentage of the total Canadian population diagnosed with diabetes is now believed to be 5% or even higher in certain groups such as the Native population. By including estimates of undiagnosed (and therefore unreported) diabetes, the estimate of all cases is believed to be between 7 and 10% of the Canadian population. Over 1.5 million Canadians have been diagnosed with diabetes. Another half of that number are believed to have diabetes but do not know it. Incidence and prevalence of diabetes increases with age. It is estimated that by the year 2004, one in four Canadians over the age of 45 will have diabetes mellitus (2).

1. Insulin-Dependent Diabetes Mellitus

The World Health Organization (WHO) designates four major clinical classes of diabetes; insulin dependent diabetes mellitus (IDDM) or Type 1 diabetes, non-insulin dependent diabetes mellitus (NIDDM) or Type 2 diabetes, malnutrition-related diabetes mellitus (MRDM), and other types of diabetes associated with certain conditions and syndromes for example pancreatic disease, disease of hormonal etiology, drug-induced or chemical induced conditions, abnormalities of insulin or its receptors, and certain genetic syndromes (3). In addition, diabetes first recognized during pregnancy is termed gestational diabetes, but such cases cannot be specifically classified as to type until postpartum period (3). This chapter will focus its discussion on IDDM.

IDDM occurs when the pancreas is unable to produce insulin. It is characterized by absolute insulin deficiency, abrupt onset of severe symptoms, proneness to ketosis and dependence on exogenous insulin to sustain life (3). IDDM is one of the most common chronic childhood illnesses, affecting an estimated 50,000 new cases per year worldwide (4, 5). It often appears suddenly in early adolescents, however, type 1 diabetes may occur at any age and affects about 10% of all people with diabetes. To manage the condition and stay alive, an individual with type 1 diabetes requires daily insulin injections, as well as healthy meal planning and regular exercise. In 1993, studies performed by the Diabetes Control and Complications Trial (DCCT) Research Group demonstrated that intensive insulin therapy to maintain blood glucose concentrations close to normal range can delay the onset and slow progression of long-term complications in IDDM (6). These complications include kidney failure, blindness, and heart failure.

2. Etiology of IDDM

IDDM results from a chronic autoimmune destruction of the pancreatic β cells, probably initiated by exposure of a genetically susceptible host to an environmental agent (7). Although the candidate genetic and environmental factors appear to be quite prevalent, β cell autoimmunity develops in less than 10% and progresses to diabetes in less than 1% of persons in the general population. The autoimmune process is mediated by macrophages and T lymphocytes with circulating autoantibodies to various β cell antigens. Epidemiologic studies have defined autoimmunity as the presence of autoantibodies (8). Earlier studies have described the epidemiology of β cell autoimmunity by measuring islet cell antibodies (ICA) in the classical immunofluorescence test using pancreatic tissue (9). This test has been very difficult to standardize and is now being replaced by combinations of several assays for antibodies against specific β cell antigens, such as insulin (IAA) (10, 11), glutamic acid decarboxylase (GAD) (12, 13), ICA 69 (14), 37-kda antigen (15), and ICA512 (16).

a) Genetic factors

In contrast to the wealth of data concerning genetic markers associated with clinical diabetes, little is known about the genetic determinants of β cell autoimmunity. No particular HLA type seems to be associated with β cell autoimmunity, although inconsistent associations between insulin or GAD autoantibodies and HLA-DR, DQ phenotypes have been reported. The HLA-DR2, DQB1*0602 haplotype, which almost completely protects from type 1 diabetes (17) is found in about 15% of GAD and IAA-positive young relatives of IDDM patients (18). However, over 90% of those strongly or persistently ICA positive are HLA-DR3 or 4, similar to IDDM patients (19). Children with β cell autoimmunity identified from the general population also show a variety of HLA genotypes, but 90% of those persistently ICA positive are DRB1*04, DQB1*030 (20, 21). This suggest that HLA genes may not be involved in the initiation of β cell autoimmunity, but rather determine progression to diabetes. More subjects with β cell autoimmunity need to be genotyped to precisely determine the role of HLA and additional type 1 diabetes candidate genes (22-26).

b) Environmental factors

i) viruses

The concept that a preceding viral infections can trigger diabetes mellitus has been given serious thought for many years (27). The idea that viruses might cause some form of IDDM comes from numerous case reports showing temporal relationship between the onset of certain viral infections (for example, rubella, mumps and Cocksackie B viruses) and subsequent development of diabetes (28-31). ICA or IAA has been detected after mumps (32), rubella, measles, and chicken pox (33), Cocksackie virus (34), and ECHO4 (35) infections. Newborns and infants are particularly more susceptible to develop a persistent infection and among patients with congenital rubella syndrome; 70% have ICA (36). The evidence is strongest for picornaviruses, which include human (enteroviruses and rhinoviruses) and animal pathogens (mouse EMC virus and Theiler's virus). Picornaviruses induce type 1 diabetes in numerous animal models, and their tropism to human β cells has been demonstrated (37). A molecular mimicry between the P2-C protein of Coxsackievirus and the GAD protein (38) may be responsible for β cell autoimmunity. Cross-sectional studies of anti-Coxsackievirus antibodies in β cell autoimmunity have been weak and inconclusive (39, 40). However, a prospective study of ICA-negative Finnish relatives found a strong relation between enteroviral infections and development of ICA or IDDM during a 3-year follow-up (41). Other case reports and epidemiological studies support the idea of virus-induced diabetes by showing a) viral specific antigens in the islets of Langerhans and destruction of β cells in the pancreas of diabetic patients (42); b) the presence of viral antibodies with rising titers in paired sera from newly diagnosed IDDM patients (42, 43); c) high frequency of Cocksackie B virus-IgM specific antibody in newly identified diabetic children (44, 45); d) β cell damage in children who died of well-documented, overwhelming viral infections (46); and e) the isolation of viruses from patients with acute-onset diabetes and the demonstration that these isolated viruses could induce diabetes in mice (42, 47).

ii) dietary factors

Exposures to cow's milk, bovine serum albumin (BSA), or β-casein prior to gut cellular tight junction closure or during gastroenteritis when the intestinal barrier is compromised are alternative causes of β cell autoimmunity. Cow's milk or wheat introduced at weaning trigger insulitis and diabetes in animal models (48), perhaps through a molecular mimicry between BSA and a rat β cell protein p69 (49). Human data are few and conflicting. A human homologue of p69 (ICA69) shows two different regions of homology with BSA (50). A study of 94 relatives (median age 5 years) did not find any association between breast feeding or early exposure to cow's milk and the presence of GAD, IAA, or ICA 69 autoantibodies (51). The role of ICA 69 in IDDM and the existence of the molecular mimicry between ICA 69 and BSA are controversial (52). Although anti-BSA antibodies are more prevalent in ICA/IAA-positive than in ICA/IAA-negative relatives of IDDM patients or unrelated controls (53), this could be explained by higher responsiveness to BSA in HLA-DR3 subjects, who account for a majority of autoimmunity cases (54). Anti-BSA antibodies are not elevated in most of the relatives followed to diabetes (54).

The association between cow's milk and autoimmunity could be due to the effect of β-casein immunostimulating hexapeptide present in enzymatic hydrolysate of milk from *Bos taurus* cows, but not from *Bos indicus* cows (7). Chemical compounds (for example streptozotocin; 55) or dietary nitrates and nitrozamines (56) induce β cell autoimmunity in animal models. Multiple hits of dietary β cell toxins may render genetically resistant individuals susceptible to diabetogenic viruses leading to IDDM (57).

An interesting hypothesis is that cow's milk might exert detrimental effects on gut or systemic immunoreactivity leading to disturbed peripheral tolerance mechanisms and/or enhanced susceptibility to viral infections (58). Upon digestion, several caseins give rise to opioid peptides, which bind to opiate receptors on immune cells leading to activation of mast cells or to modulation of T-cell and macrophage reactivity. Whereas β-casein of *Bos taurus* (allele A1) gives rise to β-casomorphin 7, β-casein of *Bos indicus* (allele A2) does not. In non-obese diabetic (NOD) mice, β-casein A1 was found to be diabetogenic when added to an elemental non-diabetogenic diet, whereas β-casein A2 was not. Furthermore, admixture of the μ-opioid antogonist naloxone suppressed the diabetogenic effect of β-casein (58). Interestingly, most of the milk produced in the Western world contains substantial amounts of β-casein A1. The consumption of A1 but not A2 per capita appears to correlate with regional (national) diabetes risk (59).

I-B) CURRENT TREATMENTS FOR IDDM

1. Subcutaneous Insulin Injection

The discovery of insulin at the University of Toronto in 1922 was one of the most dramatic events in the history of the treatment of type 1 diabetes (60). Conventional subcutaneous insulin injections have largely prevented the acute metabolic complications of IDDM, however the long-term complications, particularly micro-and macroangiopathy are still the major source of morbidity and mortality among the diabetic population. Despite close monitoring of blood glucose and the use of multiple injections of regular insulin to closely regulate blood glucose, this treatment cannot fully and constantly control metabolic dysfunction in patients with IDDM. The relative ineffectiveness of the subcutaneous insulin therapy is mainly attributable to its inability to reproduce tight reciprocal control between blood glucose and insulin levels (61). Another variable of potential importance is the site of insulin delivery, which occurs directly in the systemic circulation after subcutaneous injections, as opposed to the physiological situation, where the hormone is secreted into the portal vein and a significant portion of insulin is extracted by the liver. The subcutaneous therapy, thus, is not able to reproduce the physiologic insulin gradient between the liver and the systemic circulation, which perfuses the peripheral insulin-sensitive tissues. Consequently, insulin-treated patients will expose the liver to lower insulin levels and their peripheral tissues to higher insulin levels than the normal subjects.

An alternative approach for reaching and sustaining near-normoglycemia, as close as possible to the physiological model is the replacement of pancreas or islets by transplantation. The possibility of transplanting pancreatic tissue has interested experimental investigators since 1889 when Von Mering and Minkowski (62) demonstrated that removal of the canine pancreas resulted in hyperglycemia and suggested pancreatic transplantation in dogs. In 1893, Williams and Harsant (63) were the first to attempt islet tissue transplantation in a diabetic patient using bits of sheep pancreas.

2. Pancreas Transplantation

Early attempts at transplanting the pancreas (64, 65) were considered unsuccessful because the exocrine enzymes autodigested the transplanted tissue and injured the host (66). It was on December 16, 1966 that the first human pancreas transplant was done by Kelly, Lillehei, and associates for a uremic diabetic recipient of a simultaneous kidney transplant (67). Although graft pancreatitis developed, endocrine function was sustained for several weeks, before rejection of both organs occurred. This initial attempt clearly showed that a pancreas transplant could establish a normoglycemic, insulin-independent state for a human with type 1 diabetes. A second pancreas transplant (again simultaneous with a kidney) was done on December 31, 1966. In contrast with the first, this was a whole pancreas-duodenal-proximal jejunal allograft, and exocrine secretions were drained externally *via* a cutaneous jejunostomy (67). Another 12 pancreas transplants were done by Dr. Richard Lillehei and associates from 1967 to 1973 (68, 69), completing a series of 14 (70). Although most grafts functioned immediately (patients became insulin-independent), all except one were rejected within 1 year. The results were not as good as had been hoped and the need to go back to the drawing board was apparent by the early 1970s. As many as 7,505 pancreas transplants have been reported to the International Pancreas Transplant Registry from 1966 to 1995 by more than 170 institutions (71). Approximately 85% were in diabetic renal allograft recipients (72). Since 1986, success rates (insulin-independence for ≥1 year) as calculated by the Pancreas Transplant Registry, have been over 75% for pancreas transplants performed simultaneous with a kidney (SPK), and over 50% when performed as a solitary procedure (71). With good HLA matches, however, the success rate with bladder-drained solitary pancreas transplant has also been over 70%. With the new immunosuppressive agents available, HLA matching for solitary pancreas transplants may be less important. In a recent multi-center analysis of institutions using FK506 in pancreas transplant recipients (71), graft survival rates were over 80% for all categories of recipients (SPK, pancreas after kidney (PAK), pancreas transplant alone (PTA)). Similar results were obtained in a single center analysis (73, 74).

Although pancreas transplantation offers a strong opportunity to induce, quite reproducibly, remission of hyperglycemia in IDDM patients, one of its major drawbacks is the major surgical procedure it requires which results in a significant morbidity and a small but definite mortality (75, 76). In addition, vascularised pancreas transplant recipients require profound long-term immunosuppression with serious side effects (77, 78). Multiple-dose immunosuppressive treatment is invariably required to prevent the host's immune response, and many patients will also require either short courses of high dose corticosteroids or antilymphocyte globulin (ALG) or OKT3 for the management of acute rejection crises (70). The potential recurrence of autoimmune disease in the transplanted pancreas remains an open issue. In spite of previous observations that such an immune response can only be recognized in an identical twin receiving segmental pancreatic graft from the sibling without immunosuppression (79) there had been evidence that showed autoimmune disease may also recur in immunosuppressed IDDM patients receiving cadaveric pancreatic allografts (80). The recurrence of autoimmune response also applies to islet grafts (81), however unlike the whole pancreas, islets can be treated by a variety of methods designed to reduce their immunogenecity.

3. Islet Transplantation

Islets of Langerhans occupy only 2% of the volume of the pancreas (76). Thus the complications associated with transplanting the remaining 98% of the gland, including the exocrine cells and harmful proteolytic enzymes, may be avoided by transplanting only the isolated endocrine tissue, which is all that is required to relieve the diabetic state. Islet transplantation offers the potential advantages of pre-transplant reduction of immunogenecity thus possibly obviating the need for continuous life-long recipient immunosuppression. Moreover, islet cells can be pre-treated in culture, transplanted into immunopriveleged sites, and/or transplanted days to weeks after recipient pre-treatment with donor antigen (as outlined in Table 1-1; 76). Long-term preservation of large amounts of isolated islets can also be performed by cryopreservation techniques. Isolated islets of Langerhans derived from rodent pancreas can be frozen and stored at -196 °C for months or years without evident loss of viability (82, 83). Islet transplantation is a rather simple procedure associated with only minor risks, if any (84). In this case the application of islet transplantation may be extended to non-uremic type 1 diabetics including diabetic children which is the ultimate target group for transplantation of isolated pancreatic islets.

Table 1-1. Comparison between whole pancreas and islet transplantation.

	Whole pancreas	Islet
GRAFT		
Preparation	Surgery	Surgery+Laboratory
Preservation	<24h	>24h
Quality control	Not possible	Possible
Tissue pretreatment	Not possible	Possible
Tissue banking	Not possible	Possible
IMPLANTATION		
Technique	Complex	Simple
Site	Normal	Ectopic

(From: Purello F, Pipeleers D. Transplantation in diabetes: a cell biological problem. J Endocrinol Invest 1995; 18: 311-319).

Pancreatic islets were first described by Paul Langerhans in 1869, when his thesis as a medical student in Virchow's laboratory was published (85). Following the successful autologous transplantation of pancreatic fragments in a dog by Minkowski in 1892 (86) in an attempt to prevent the development of diabetes mellitus, the idea of separating islet tissue from the exocrine tissue of the pancreas before transplantation was suggested by Ssobolew (87). The message of Ssobolew was not well taken and it took sixty years until the first method was described to isolate islets *via* hand dissection under the microscope (88). This method was traumatic and yielded only a small numbers of islets per pancreas. The first major contribution to islet transplantation was made by Moskalewski (89) who used the enzyme collagenase to digest chopped guinea pig pancreas. Lacy and Kostianovsky (90) improved the technique by intraductal distention of the pancreas to disrupt the exocrine tissue prior to mechanical mincing and enzymatic digestion of the gland. The islets were then separated from the digested pancreas by centrifugation in sucrose gradients. These modifications resulted in isolation of approximately 300 intact islets from a single rat pancreas. Later on centrifugation on Ficoll gradients proved to be most effective (compared to sucrose) since it provided a better osmotic environment for the islets (91, 92).

The first report of transplanting isolated rodent islets was in 1970 by Younoszai and associates (93). They demonstrated temporary amelioration of chemically induced diabetes in rats. Ballinger and Lacy (94) were the first to demonstrate a sustained and significant reduction of hyperglycemia (as measured by urine volumes, urine glucose and weight gain) by transplanting 400-600 isolated islets to the peritoneal cavity or thigh muscle of inbred diabetic Lewis rats. In 1973, Reckard and Barker (95) demonstrated complete normalization of glucose in rats for as long as 7 months following intraperitoneal injection of 800-1,200 autologous islets. Kemp and co-workers (96) demonstrated that the same number of islets, approximately 800 (that only partially ameliorated the diabetic state) could completely normalize plasma insulin and glucose concentrations when embolized to the liver by the portal vein. The physiological significance of the hepatic portal circulation has been demonstrated by a number of investigators (97-100). As well as the intraperitoneal (93-95, 101-103) and intraportal sites (96, 97, 103-105), islets have been transplanted to the spleen (100, 106, 107), a surgically created peritoneal-omental pouch (108), the renal subcapsular area (98-100, 106-111), the testicles (112), the ventricles of the brain (113), and the thymus (114). With respect to transplantation site, the most consistent results occurred when the graft is placed in a highly vascular bed with portal venous drainage.

The experimental success of islet transplantation in rodents prompted studies in larger animals, including primates, with the idea of applying islet transplantation to the treatment of diabetes in humans. It soon became apparent that the standardized rat isolation techniques would not work in the more compact and fibrous mammalian pancreas, in particular the human pancreas (105-120). The yield of islets from one human donor was insufficient to ameliorate the diabetic state. Unlike rodents, multiple donors could not be used in a population of outbred large animals as allogeneic islets would be rejected before function of a technically successful graft could be determined. Mirkovitch and Campiche (116) partially solved the problem of islet yield by eliminating the purification step (density gradient separation) used in the rodent model. They reversed diabetes in 20 of 25 dogs with intrasplenic autografts of dispersed pancreatic tissue prepared from the tail of the gland. Kretschmer and associates (117) demonstrated that normoglycemia occurred in 20 of 21 dogs receiving intrasplenic autografts of dispersed pancreatic fragments prepared from the entire gland. Preparation of the grafts involved mincing the entire pancreas in a mechanical tissue chopper followed by collagenase digestion in a shaking water bath. Using these techniques, or modifications thereof, autografts of dispersed pancreatic fragments prepared by enzymatic digestion and mechanical dispersion have been shown by other investigators to reverse the diabetic state in dogs (121-126).

After years of research developing methods to isolate islets from large animals and exploring different transplant sites, it finally became possible to begin clinical islet transplantation in the late 1980s. A total of 305 adult islet allotransplants were performed for patients with type 1 diabetes at 35 institutions worldwide between 1974 and 1996; 215 such transplants were performed at 27 institutions between 1990 and 1996 (127). More than 60% of these were transplanted at 6 institutions , including 55 at the University of Minnesota; 34 at the University of Giessen; 27 at Washington University; 26 at the University of Pittsburgh; 22 at the University of Miami; 21 at the Institute H. San Raffaele; and 7 at the University of Alberta. Insulin independence for more than one week was documented in a total of 33 (10.8%) of the 305 recipients transplanted between 1974 and 1996 and in 30 (14.0%) of the 215 recipients transplanted between 1990 and 1996. The number of recipients who remained insulin-independent for more than 4, 3, 2, and 1 year(s) is 1 (0.5%), 4 (1.9%), 7 (3.3%), and 13 (6.0%), respectively. The recipient with the longest (>5 years) insulin-independent follow-up was transplanted at the Washington University and continues to sustain euglycemia in the absence of exogenous insulin (128).

Insulin independence after single-donor (1:1) islet allotransplantation was reported in 16 of the 33 recipients mentioned above; 5 recipients achieved insulin independence after 2:1; 5 after 3:1; and 7 after >3:1 donor-recipient islet transplants. Insulin independence was achieved in 16 recipients of an islet-after-kidney (IAK) transplant, in 14 recipients of a simultaneous islet-kidney (SIK) transplant, in 1 recipient of a simultaneous islet-liver (SIL) transplant (127), in 1 non-uremic recipient of an islet transplant alone (ITA) (129), and in 1 recipient of an intraportal islet allograft prepared from a previously transplanted pancreas after graftectomy (130). Well-matched single-donor islet allografts have rarely been performed and none of the 16 type 1 diabetic patients who became insulin-independent after single donor-islet allotransplants received a 5- or 6-antigen matched islet graft which suggests that HLA matching might not be a critical factor in islet allograft survival.

Of the 33 insulin-independent recipients between 1974 and 1996, 25 had no residual β cell function pre-transplants as demonstrated by basal or stimulated plasma C-peptide levels ≤ 0.2 ng/ml. Six had undergone a previous pancreas transplant, two a previous islet transplant. On average, 11,118±1,152 islet equivalents/kg were required to establish insulin independence (127). The purity of the islet graft varied from 5% to 98%. Moreover, in 30 of the 33 insulin-independent recipients, islets were transplanted into the liver; 1 recipient received an intrasplenic islet allograft; in another islets were transplanted into an epiploic flap; and in another, encapsulated islets were injected intraperitoneally (131, 132). Twenty nine of the 33 insulin-independent recipients received monoclonal or polyclonal T-cell antibodies for induction immunosuppression. Maintenance immunosuppression included steroids in all but 5 of the 33 insulin-independent recipients. In all but four, cyclosporine was the mainstay of maintenance immunosuppression; in 1, tacrolimus (FK506). Triple-drug maintenance immunosuppression was used in 25 of the 33 recipients; of those 25, 22 received azathioprine and 3 mycophenolate mofetil. The average time to achieve insulin independence was 179±24 days. The mean duration of insulin independence was >443±78 days. A total of 10 recipients are still insulin-independent as of July 1, 1998 (127). The results of above series of clinical investigations established the minimal requirements (as outlined in Table 1-2; 84) that are critical for the preparation of islets intended for clinical islet transplantations.

Table 1-2. Minimal requirements to be met by islet preparations intended for clinical islet transplantations.

• Islet Mass	>8,000 IEQ/kg body weight in type 1 diabetic recipient >3,500IEQ/kg body weight in patients to be autotransplanted after pancreatectomy
• Islet Purity	>80% (percentage of the islet volume in the total cell volume)
• Islet Viability	>80% (assessed by a microfluorometric membrane integrity test)
• Islet *in vitro* function	Documented biphasic response to a glucose challenge and return to baseline phenomenon
• Islet culture	Exclusion of microbial contamination

(From: Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabetes mellitus - from bench to bedside. Exp Clin Endocr Diab 1995; 103: 143-159).

I-C) BARRIERS TO SUCCESS OF ISLET TRANSPLANTATION

1. Limited Supply of Human Islet Tissue

A major barrier to the future success of islet transplantation is the limited availability of human islet tissue. Because the demand for intact and viable pancreatic donor tissue intended for islet transplantation already exceeds the availability, new sources for transplantable islet tissue are drastically needed. Each year in the US, approximately 5,000 brain-dead donors with intact circulation become available, and only a portion of these are suitable for islet or pancreas transplantation (133). Yet there are approximately 30,000 new cases of type 1 diabetes each year (134), not to mention the huge number of people with type 2 diabetes, some of whom might be candidates for transplants since there is not enough insulin produced by the pancreas to meet the body's requirements (as opposed to those who cannot use the insulin that is produced by the pancreas). The possibility of using islets donated by living donors is not an attractive option, in part because of the risk of the donor developing diabetes (135).

The limitation in the supply of adult islet tissue could theoretically be alleviated by exploiting the growth capacity of fetal tissue, however, the potential of human fetal endocrine pancreas is clouded by a variety of political and ethical issues (136). While nonhuman primates might be preferred as a source of islets for diabetic patients, the number of full-sized primate pancreas that could be obtained is far short of the number needed (137). Thus, a variety of animal species have been considered as islet donors, but pigs and cows, which are already used commercially for food, seem to hold the most promise. Pigs seem to be appealing for a variety of reasons. First, many physiologic similarities exist between swine and humans (138). Pigs are omnivores, and their glucose levels are similar to those of humans. Second, pigs are used in large numbers on a daily basis for human consumption. Pancreas is a waste product of the slaughtering process thus, no ethical problems should result from the utilization of porcine islet donors. In addition, pigs may be raised in controlled environment, thus making the use of their organs/tissues as xenografts more acceptable. Pigs can be subjected to genetic manipulation, which means that transgenic pigs can be developed with genes expressed in their β cells that could help resist immune attack and even enhance insulin secretion (139). Third, a product of the pig pancreas, *i.e.*, porcine insulin, differs from human insulin by only one amino acid and has been routinely and safely used to treat diabetic patients for decades.

a) Adult porcine islets

The first description of a method for the isolation of porcine islets appeared in 1974. Sutherland *et al.* (118) use a modification of the collagenase technique described for rat isolation to isolate porcine islet tissue and used the material for autotransplantation studies in pigs. No evidence of function was obtained from the autografts, and no information was provided on the morphologic and functional integrity or degree of purity of the islet preparation obtained by this method. In 1986, Ricordi *et al.* (140) reported that an average of 79, 000±6,028 islets per pancreas can be obtained from adult pigs by an enzymatic and mechanical procedure. The viability of the islets was tested by glucose stimulation *in vitro* (140) and by transplantation in diabetic mice (141). Marchetti *et al.* (142) were able to isolate an average of 500, 000 islets per pig pancreas, using a method based on collagenase digestion and filtration of the digested tissue. No purification process was used, and the islets showed morphologic integrity at histologic examination. The purity of the final preparation was 10% to 20% islets. The following year, Hesse *et al.* (143) documented results after islet autotransplantation in pigs, using an isolation method that was derived from the dog model (144). No information was provided on quantitative and qualitative assessment of the islet preparations before autotransplantation, and most of the preparations were not purified at the time of transplantation. The high incidence of complications in which an unpurified preparation was used has led to a number of studies in developing new isolation method to improve the quality and quantity of isolated islets (145-150). However, the task has never been easy because the quality and numbers of islets that can be obtained from adult pigs are very much dependent on the age, breed, weight, and diet of the animals (150-154). In addition, adult pig islets are fragile and difficult to maintain in tissue culture (149). Adult pig islets are often irregular in shape and lack an outer capsule membrane. Moreover, peripheral islet cells in adult pig islets are directly apposed to acinar cells and loose vascular channels are present within the islet making it easy for these tissues to fragment during isolation procedure (145). Warnock *et al.* (149) have found that remarkable purity can be obtained from their isolation but majority of the adult pig islets were lost during the purification stage and these losses was found to amount to 95% of the islet mass. In addition, during consecutive isolations an outstanding yield could be achieved in one, while in others the yield are very poor, suggesting some inherent variability in the donors. In spite of the advances made in the isolation protocols of adult pig islets, there are still serious problems in obtaining healthy adult pig islets and new measures must be sought to solve these problems before these tissues could be acknowledge as a potential source of insulin-producing tissue for transplantation.

b) Fetal porcine islets

The potential use of porcine fetal pancreas tissue is attractive because of the capacity for growth and the ease of maintaining sterility. In addition, the procedure for obtaining this tissue is less traumatic than that used for adult pancreases, so the cells are hardier when placed into culture or transplanted (136). These fetal pancreatic cell preparations are very complex; fortunately, the exocrine cells spontaneously die off when cultured or transplanted, but the surviving population consists of a mixture of mesenchymal, precursor, protodifferentiated, and mature islet cells. The use of fetal porcine islets for treating patients with type 1 diabetes was performed by Groth and associates in Sweden (155). Although there was no evidence to indicate engraftment of the fetal cells, all patients in this study tolerated the procedure well and no adverse effects were recorded. Unlike adult pig islets, tissue culture of collagenase digested fetal porcine pancreas produces viable islet-like cell clusters (156, 157), which have the ability to cure diabetes in nude mice within two months post-transplantation (156). A general finding, however, in rat (158-161), porcine (156), and human fetal pancreatic β cells is that they exhibit a poor insulin secretory response to glucose, and the onset of maturation of glucose-induced insulin secretion is more evident in the post-natal period (158-161). Thus, recent attention has been paid to the potential of pancreatic cells obtained from the neonatal pigs (162).

c) Neonatal porcine islets

Neonatal porcine islets (NPI), in particular have the advantage of being more mature than the fetal islets and yet maintaining considerable capacity for growth. Korbutt *et al.* (162) has developed a simple, standardized procedure for isolating large numbers of neonatal porcine islets with a reproducible and defined cellular composition. After 9 days of culture, the average number of neonatal porcine islets recovered from one pancreas was approximately 50,000 (range = 28,210-90, 966). As opposed to adult pig islets which are known to consist of approximately 80-90% endocrine cells, neonatal porcine islets consist primarily of 35% fully differentiated endocrine cells and 57% epithelial cells or endocrine precursor cells (162). *In vitro* viability assessment of the cultured islets showed that in the presence of 20 mM glucose, the islets were capable of releasing 7-fold more insulin than at 2.8 mM glucose. When exposed to 20 mM glucose plus 10 mM theophylline, the stimulation index increased to 30-fold compared to basal release. Moreover, transplantation of 2,000 neonatal porcine islets (consisting of $6x10^5$ β cells) under the kidney capsule of alloxan-induced diabetic nude mice corrected hyperglycemia in 100% (20/20) of recipients within 8 weeks post-transplantation and remained euglycemic until the graft-bearing kidney was removed at 100 days post-transplantation (Figure 1-1; 162). Examination of the neonatal porcine islet grafts revealed that they were largely composed of insulin-positive cells (Figure 1-1; 162) and the cellular insulin content of these grafts was 20 to 30-fold higher than at the time of transplantation. These results indicate that abundant NPI which have the potential for growth can be routinely isolated in the laboratory and may possibly be used for clinical transplantation.

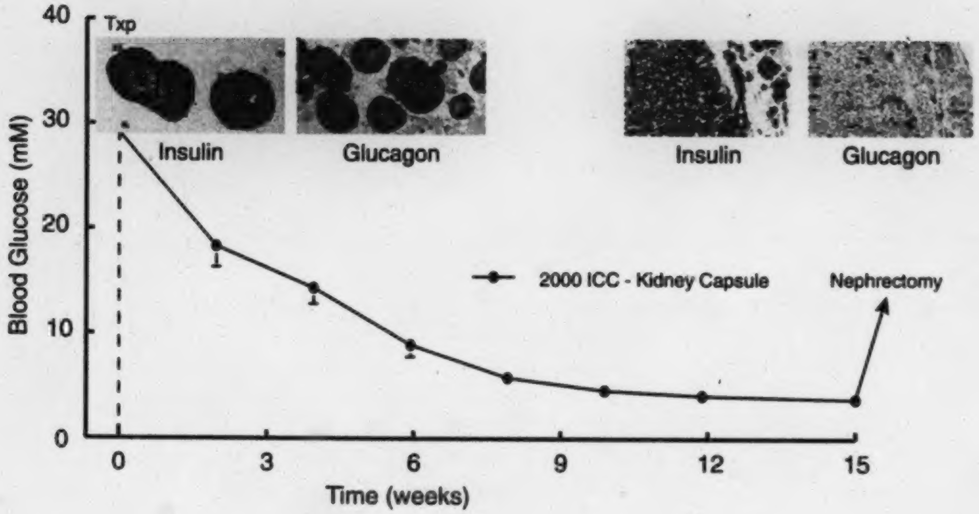

Figure 1-1. Blood glucose levels of diabetic nude mice transplanted with NPI. Inserts are light micrographs of NPI (before and after transplantation) stained for insulin and glucagon. (From: Korbutt *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-2129).

2. Immunological Responses to Neonatal Porcine Islets

Before NPI can be transplanted to humans, the immunological responses to these tissues must first be examined.

a) Humoral-mediated immune response

i) hyperacute rejection

A major barrier to discordant (*e.g.* pig-to-human) xenotransplantation of whole organs is the occurrence of hyperacute rejection (HAR), a process believed to be initiated when naturally occurring xenoreactive antibodies in the recipients sera binds to antigens present on the surface of endothelial (and other) cells within the xenograft. Antibody binding in turn activates complement, which rapidly destroys the transplanted organ or tissue within minutes to few hours (163-167). HAR is characterized pathologically by interstitial hemorrhage and diffuse thrombosis. It occurs nearly always in some combinations of donor and recipient species, but rarely in others, these combinations being called "discordant" and "concordant", respectively (164, 168). The susceptibility of a given combination to HAR was thought to have a genetic basis, reflecting at least in part the phylogenetic distance between the donor and the recipient (168-171). During the past seven years, the molecular mechanisms underlying the susceptibility of porcine organs to HAR by humans or nonhuman primates, and the basis for the discordance in these species combinations, have been shown to be related to two primary factors: (1) the binding of xenoreactive natural antibodies (XNA) of the recipient to antigens on endothelial cells in the graft; and (2) the incompatibility of complement regulatory proteins in the transplant with the complement system of the recipients, allowing uncontrolled activation of complement (137).

Upon binding to porcine endothelium, IgM XNA activate the complement cascade (166, 172) and also directly activate endothelial cells to an inflammatory state (173). Endothelial cell cytolysis induced by fresh serum is commonly observed *in vitro* (172-174). Because the integrity of the endothelium is lost (174) and thrombosis is promoted (173), IgM XNA plays a crucial role in the triggering of hyperacute xenograft rejection. The role of IgG XNA is not well established. It has been reported that IgG XNA do not activate complement *in vitro* (166, 175, 176) although some authors disagree (179). IgG

XNA are, however, able to cooperate with NK cells *in vitro* to kill porcine endothelial cells through an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism (180-182). Alteration of the hemocompatible endothelial barrier by this mechanism could initiate an early delayed vascular rejection (180). The IgG XNA-dependent NK cell-mediated cytotoxicity against porcine aortic endothelial cells (PAEC) is absolutely dependent upon FcγRIIIA (CD16A), as demonstrated by blocking experiments (181, 183).

While one might imagine that human XNA would recognize a wide array of porcine antigens, greater than 80% of the complement-fixing XNA in a human serum recognize a single structure, galactoseα(1,3)galactose or Galα(1,3)Gal (184). Anti-Galα(1,3)Gal antibodies develop in humans and higher primates within the first months of life in parallel with the colonization of the gastrointestinal tract with bacteria containing α(1,3)-linked galactose residues in their cell walls (185); consequently, there exists a window period in which these IgM antibodies are not present in neonatal primates (186). The absence of preformed IgM anti-Galα(1,3)Gal antibodies in neonatal primates enables porcine cardiac xenografts transplanted heterotopically into unmedicated newborn baboons to survive beyond the hyperacute period (187-189), making this an appropriate model for studying the subsequent immunologic barriers to xenotransplantation. In these recipients, a second primate anti-porcine immunologic response occurs after 3-4 days, resulting in graft loss accompanied by dense xenograft infiltration with monocytes/macrophages, natural killer (NK) cells, and deposition of induced IgG antibodies (190).

ii) Galα(1,3)Gal antigen

The most important target for human xenoreactive antibodies has been identified as the terminal carbohydrate Galα(1,3)Gal epitope (191, 192, 193). The Galα(1,3)Gal carbohydrate residue is present in high concentrations on all porcine endothelial cells (194-196) and has been detected on fetal porcine islet cells (197, 198). Species such as pigs, phylogenetically distant from human beings, express an α1,3-galactosyltransferase (α1,3GT) that catalyzes the transfer of an α-galactosyl residue to a terminal β-galactose leading to the oligosaccharide Galα(1,3)Galβ(1,4)GlcNAcβ-R, which is present on all cell surface glycoproteins and glycolipids (191, 198). The α1,3GT is present in all mammals except humans, apes, and Old World monkeys (Figure 1-2; 199), in which the α1,3-GT gene has been inactivated as the result of frame shift and nonsense mutations (186). Humans therefore develop antibodies to Galα(1,3)Gal as a result of exposure to Galα(1,3)Gal on the surface of enteric bacteria and other pathogens (191).

The importance of Galα(1,3)Gal as a target of human xenoreactive antibodies was initially demonstrated by carbohydrate competition studies, in which it was shown that only carbohydrates containing α-galactose residues were capable of blocking human xenoantibody reactivity with porcine endothelial cells (200, 201). These studies were confirmed by Sandrin' s group (202), who demostrated that transfection of COS cells (Galα(1,3)Gal-negative cell line from Old World monkey origin), with cDNA for α1,3-GT cause *de novo* expression of Galα(1,3)Gal and rendered these cells sensitive to human serum-mediated cytotoxicity. Furthermore, absorption of human serum against α1,3-GT-transfected COS cells stripped the serum of xenoantibodies capable of reacting with porcine endothelial cells. Finally, Collins *et al.* (203) demonstrated that elimination of Galα(1,3)Gal from porcine cells rendered these cells resistant to human xenoreactive antibodies and complement.

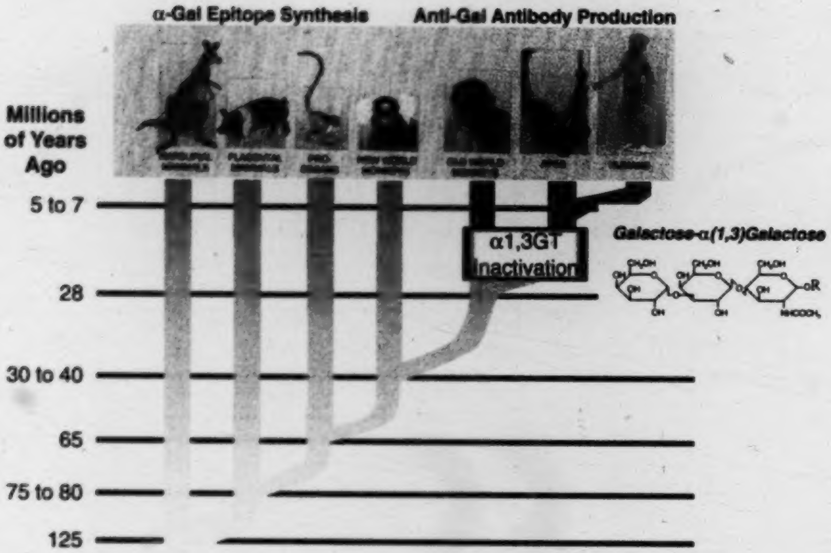
α-Gal Epitope Synthesis
Anti-Gal Antibody Production
Millions of Years Ago
5 to 7
28
30 to 40
65
75 to 80
125
α1,3GT Inactivation
Galactose-α(1,3)Galactose
CH₂OH
OH
-oR
NHCOCH₃

Figure 1-2. Reciprocal evolution of Galα(1,3)Gal epitopes and the anti-Gal antibody in mammals. The Galα(1,3)Gal epitope has been produced in mammals by α(1,3)galactosyltransferase (α1,3GT) for more than 125 million years, since before the divergence of placentals and marsupials. Non-mammalian vertebrates all lack α1,3GT and do not produce Galα(1,3)Gal epitopes. Production of this epitope was suppressed in ancestral Old World primates after they diverged from New World monkeys, and probably after apes and monkeys diverged from each other. Suppression of Galα(1,3)Gal was followed by production of the natural anti-Gal antibody, which is not made in non-primate mammals, prosimians, or New World monkeys. (From: Galili U. Anti-gal antibody prevents xenotransplantation. Science & Medicine 1998; 5: 28-37).

While it is now widely accepted that expression of Galα(1,3)Gal is necessary for the binding of xenoreactive antibodies to a porcine cell, some studies suggest that it is not sufficient. For example, Platt *et al.* (204) found that porcine integrins with Galα(1,3)Gal substitutions block antibody binding far more effectively than some other proteins that have more Galα(1,3)Gal. Alvarado *et al.* (205) demonstrated that a seven-fold range in binding of human anti-Galα(1,3)Gal antibodies to cells from a large population of pigs is not related to differences in cell-surface Galα(1,3)Gal. These observations suggest that the binding of XNA to a cell surface is influenced to a large extent by three dimensional array of epitopes, which depends on the nature of the protein core and its positioning on the cell surface.

The HAR phenomenon was speculated to be less of a problem with islet transplantation because islet cells seem to have very little of the Galα(1,3)Gal epitope (206) and because the vascularization of transplanted islets seems to come entirely from recipient endothelial cells (207). HAR was also suggested to be more of a problem for fetal or neonatal pancreas cells because duct cells, which are precursor cells for islet cell formation, appear to express the Galα(1,3)Gal epitope (208).

The xenograft rejection process seems far more complex than just Galα(1,3)Gal epitope-dependent HAR because there appear to be other antibody- and complement-mediated assaults that provide a serious challenge to the success of these transplants (209, 210). Several investigators in both small and large animal models have shown that HAR can be prevented through the depletion or inhibition of the recipient complement system. However, graft loss still occurs after several days (211). This process of rejection is referred to as acute vascular rejection (AVR) or delayed xenograft rejection (DXR). The most extensive study of AVR has been performed in the guinea pig-to-rat heart transplant model. AVR in this model has pathological similarities to HAR. Both types of rejection are characterized by vascular changes, including interstitial edema, hemorrhage, and fibrin accumulation (212). However, AVR differs from HAR in that a prominent infiltration of monocytes and NK cells and a widespread interstitial deposition of fibrin develops over the 72-96-hour engraftment period. Saadi *et al.* (213) reported that deposition of xenoreactive antibody and the formation of the membrane attack complex on porcine aortic endothelial cells lead to the induction of interleukin (IL)-1α and this cytokine appears to function in an autocrine or paracrine fashion to promote at least some of the changes in endothelial cells. This probelem may be exacerbated by vasoconstriction and microvascular thrombosis, resulting in vascular stasis, where small amounts of endothelial cell-derived IL-1α would remain in the microenvironment creating a positive feedback loop leading to furthe endothelial cell stimulation (214). If IgM-XNA and IgG-XNA, as well as elicited antibodies, are depleted or inhibited, it might be expected that NK cells and monocytes would not attach to the donor organ endothelial cells, assuming that the only mechanism of binding is *via* their Fc receptors to Ig bound to the endothelial cells. However, human (or rat) NK cells may be able to directly bind to porcine (or guinea pig) endothelial cells based on specific recognition of those endothelial cells without requiring participation by IgG (215, 216). In addition, if endothelial cells are activated in the absence of XNA and complement, these cells may bind *via* ligands newly expressed on the endothelial cells. It has been shown that NK cells can damage endothelial cells (216, 217); it is not unlikely that activated monocytes would also lead to endothelial cells activation and/or damage. Monocytes are also capable of expressing tissue factor and under certain circumstances a prothrombinase complex, as well as synthesizing complement components (218). Thus, the action of these cells in the absence of XNA and complement may lead to endothelial damge that would potentiate the inflammatory and procoagulant phenotype.

b) T cell-mediated immune response

It is generally accepted that two signals are needed for T cell activation, engagement of the antigen-specific T cell receptor and a second non-antigen specific inductive stimulus, or costimulator, provided by a metabolically active antigen-presenting cell (APC). Two pathways of graft antigen presentation would fulfill this two-signal requirement for T cell activation: 1) direct pathway, where host T cells recognize antigen on the surface of donor APC that are capable of costimulatory activity (donor APC-dependent), and 2) indirect pathway, whereby host T cells recognize graft antigens which are processed and presented by host APC (host APC-dependent). In allograft rejection, both pathways are believed to play a role although the direct pathway is perhaps most crucial initially, whereas, during xenograft rejection the indirect pathway is thought to be dominant. Gill *et al.* (219, 220) have demonstrated that elimination of donor APC from mouse islet allografts led to indefinite survival, while similar treatment of concordant rat-to-mouse) islet xenografts had little benefit for survival. Furthermore, rejection of the islet xenografts showed a specific dependence on CD4+ T cells, and not to the presence of donor APC and host CD8+ cells (220). It has also been shown that marked prolongation of islet xenografts can be achieved in adult pig-to-mouse model by the administration of anti-CD4 antibody (221). Islet xenograft rejection may therefore occur predominantly through the processing and presentation of xenogeneic antigens in association with class II MHC molecules of host APC, leading to a CD4-dependent inflammatory response-where other cells (*i.e.* macrophages and NK cells) recruited by this response, may also contribute to rejection. It has also been shown in skin xenograft model that removal of mouse CD4+ T cells with anti-CD4 antibody can prolong monkey or rabbit skin xenograft survival even after whole MHC-disparate allogeneic mouse skin had been rejected (222). Other studies also showed that islet allografts from transgenic mice that are deficient in class I MHC expression survived long-term as allografts but are acutely rejected as xenografts (223, 224). The role of direct antigen presentation in xenograft rejection appears to be minor, however, one must not neglect the possibility that direct recognition may also contribute to cell-mediated destruction of xenogeneic cells. Work by Murray *et al.* (225) has demonstrated that human T cells can recognize porcine endothelial cells directly and Yamada *et al.* (226) recently observed that human T cells that recognize porcine class II MHC antigens through the direct pathway can discriminate among swine leukocyte antigens (SLA) in a manner that allows for xenospecific responses.

I-D) STRATEGIES FOR PREVENTING REJECTION OF NEONATAL PORCINE ISLETS

1. Humoral-Mediated Rejection

Several approaches have been used to prevent problem of complement-mediated HAR of porcine xenografts caused by preformed antibodies directed against Galα(1,3)Gal. This immune response may be overcome by circulating human blood plasma over Galα(1,3)Gal immunoadsorbent columns to remove anti-Gal antibodies (227), however, this procedure is transient since anti-Galα(1,3)Gal antibody levels quickly return to normal in the blood (228). Another method is to inhibit triggering of the complement cascade by treatment with excess amounts of a soluble form of complement receptor (229-231). A third strategy is to generate transgenic pigs that over express α(1,2)-fucosyltransferase that competes with the substrate for Galα(1,3)Gal attachment so that a fucose group, representing a natural human blood group antigen, is substituted for Galα(1,3)Gal (232-236). Reducing the level of Galα(1,3)Gal expression on porcine endothelium by high level expression of the enzyme α(1,2)-fucosyltransferase (H-transferase) was based on the knowledge that both α(1,3)GT and α(1,2)-fucosyltransferase use the same acceptor substrate Galβ(1,4)-GlcNAc (236). α(1,2)-fucosyltransferase has preferential access however, as a result of specific amino acid sequences in its cytoplasmic domain that target its localization to particular compartments within the Golgi apparatus. Theoretically, the consequent high-level surface expression of terminal Fucα(1,2)Gal residues (instead of Galα(1,3)Gal) should not induce complement-mediated HAR of porcine xenografts, as primates do not possess preformed antibodies against this structure. A fourth approach is to knock out the pig gene that encodes α1-3GT (237, 238). This would presumably eliminate the main cause of HAR, but the technology for porcine knockouts is not yet available (228). At present, it is not possible to generate pigs lacking the Galα(1,3)Gal epitope because porcine pluripotent cells, which are essential for gene inactivation, are not yet available (238). The fifth strategy is to generate transgenic pigs that express human complement-regulatory proteins (239-246) such as CD55 (decay accelerating factor, DAF), CD46 (membrane cofactor protein, MCP-1) and CD59 (protectin). These proteins do not prevent anti-Galα(1,3)Gal antibody binding to endothelial cells to trigger the classical pathway of complement activation; rather they inhibit downstream steps in the complement cascade so that cell lysis may be prevented (228). Several transgenic pig herds have been developed that express one or more of the human genes encoding CD55, CD46 and CD59 (241, 244-247). These transgenic pigs include Large White/Landrace cross-pigs which were produced by intrazygotal pronuclear microinjection of 6.5 kb human CD55-DNA minigene construct or by sperm-mediated gene transfer of 6.75 kb DNA construct containing complete human DAF minigene (246). Pascher *et al.* (247) demonstrated that transgenic expression of the human complement regulatory protein human CD55 reduces complement activation and prevents HAR in a xenogeneic liver perfusion model over the 3-hour evaluation period.

2. Cell-Mediated Rejection

Whether xenoantigens are presented directly or indirectly by APC, a requirement for a costimulatory signal exists in both pathways. Thus, one current immunosuppressive strategy to overcome cell-mediated destruction of islet grafts is blocking costimulatory signal by treatment with the soluble protein CTLA4Ig. CTLA4Ig is a homolog of the CD28 antigen and it binds to members of the B7 family with a 20-fold greater affinity than CD28. This molecule effectively blocks the costimulatory second signal emanating from the CD28-B7 interaction (248). *In vitro* studies have demonstrated that CTLA4Ig can block B7-dependent immune responses, which results in donor-specific anergy. *In vivo* administration of this long-lived fusion protein can prevent the rejection of xenogeneic islets (248-250).

Since the late 1980's a number of new immunosuppressive agents with novel mechanisms of action and distinct therapeutic efficacies have emerged (251-261) in an attempt to overcome the cell-mediated graft rejection. The extensive experience gained with the use of immunosuppressive drugs to treat allograft rejection has been very useful for the prevention of cell-mediated xenograft rejection. Cyclosporine A (CsA) is the most widely used immunosuppressive agent in clinical transplantation. This fungal metabolite is the first immunosuppressive agent to selectively act only on a lomited population of lymphocytes and to be devoid of myelotoxicity (262). However, the use of CsA is limited by a number of side effects, the most serious of which is nephrotoxicity. Azathioprine and steroids are the other immunosuppressive agents currently used. Azathioprine is an inhibitor of RNA synthesis and as a consequence it blocks the proliferative response of sensitized lymphocytes. The main side effects of azathioprine is bone marrow depression, and this to a large extent dose-related (263) Steroids like prednisone and prednisolone have a marked lymphocytotoxic and anti-inflammatory activity. They depress the immune response in a number of ways, in particular by blocking lymphocyte proliferation probably by preventing activation of the cytokine IL-1 gene (264). Hypertension, sodium and fluid retention, pathologic fracture of long bones, pancreatitis, and suppression of growth in children are among the many adverse effects of steroids. Although present-day immunosuppression is dangerous due to its various side effects and narrow therapeutic window, extraordinary advances are being made in the field of immunology that should lead to the development of more selective and safer approaches. Glucocorticoids are particularly toxic and have adverse side effects on islet function (257), so there is hope that some steroid-sparing regimens, using such promising drugs such as 15-deoxyspergualin, leflunomide, mycophenolate mofetil, and sirolimus (rapamycin), will turn out to be useful. In addition, several immunosuppressive agents have been shown to dramatically prolong xenograft survival and have toxicity profiles compatible with their future use in clinical xenotransplantation (259). Immune reactions against islets may differ from those found with solid organ transplants and thus may require specially tailored drug regimens.

Transplantation of islets into immunopriveleged sites such as the thymus (114, 265, 266) and testes (112) is another strategy that has been used to avoid cell-mediated rejection of islet allografts. Islets transplanted into the abdominal testis has been shown to protect islets from immune destruction without the requirement of systemic immunosuppression (267-270). Sertoli cells are thought to confer immuneprivilege to the testis, possibly by producing factors that act locally to inhibit immune responses. Expression of Fas (CD95) ligand in the testes has been shown to be responsible in part for maintaining the immunopriveleged status of this site (271, 272). Fas receptor (FasR) and its ligand (FasL) are components of the immune system that play a role in killing activated T cells. Binding of FasL to FasR-bearing activated T cells leads to apoptosis of these cells (273-275). More recently, it has been shown that allografts consisting of purified Sertoli cells alone can survive long-term in immune-competent mice. Korbutt *et al.* (276) have also demonstrated that long-term graft survival of allogeneic rat islets without systemic immunosuppression can be achieved by co-transplantation of allogeneic islets with allogeneic testicular cell aggregates. Transplantation of a sufficient quantity of testicular aggregates (containing 11×10^6 cells, 75% Sertoli cells), together with 2,000 purified Lewis rat islets, reversed the diabetic state for >95 days in 100% of the streptozotocin-diabetic Wistar-Furth recipients. Similar grafts consisting of islets alone or islets plus 50% fewer testicular cell aggregates survived for only 10 days. Functioning composite allografts harvested from normoglycemic animals at approximately 100 days showed healthy β cells in close association with Fas L-expressing Sertoli cells (276).

The effect of co-transplantation of neonatal porcine Sertoli cells on the survival of NPI xenografts is not known and still under investigation. Preliminary results from our laboratory however, indicate that neonatal porcine Sertoli cells can survive under the kidney capsule in Balb/c mice for periods of 6 weeks without immunosuppression. Sertoli cells are not only responsible for the synthesis of a wide variety of proteins and hormones required for the orderly differentiation of sperm cells, they also secrete a number of immunosuppressive agents (277) such as clusterin. This is a heterodimeric glycoprotein found in a wide variety of tissues under a number of different names such as sulfated glycoprotein-2 (SGP-2), SP-40, 40, Apo J, and gpIII (278). This 70-80 kDa protein is the most abundant protein synthesized by Sertoli cells and its exact function is not known. However, clusterin has been identified as a component of circulating cytolytically inactive terminal complement complex and appears to associate with C5b67 and C8 and C9 to form clusterin-C5b-9, which is analogous to SC5b-9 (279). This protein was shown to inhibit complement-mediated cell lysis (280). Clusterin has also recently been shown to accelerate the formation of immune complexes *in vitro* which suggests that the protein has a binding site for immunoglobulins (281). From these findings, it has been speculated that clusterin may protect sperm from complement-mediated attack. In addition to clusterin, Sertoli cells also secrete cytokines such as transforming-growth factor-β (TGF-β) which is a multi-regulatory molecule that can stimulate or inhibit aspects of cellular growth and differentiation (282). TGF-β also antagonizes many responses of lymphocytes by counteracting the effects of pro-inflammatory cytokines which may be beneficial to the protection of neonatal porcine islet xenografts from T cell-mediated destruction. With this idea in mind, co-transplantation of neonatal porcine islets with neonatal porcine Sertoli cells may be a useful method in preventing both antibody/complement and T cell-mediated destruction of islet xenografts.

Immunoisolation device has also been shown to protect islets from damage by immune cells. The principle of this device is that the permeability of the membrane would be open enough to allow nutrients and oxygen to reach the islets and for insulin to be released into the bloodstream, but restrictive enough to exclude immune cells and even antibodies (283, 284). Remarkably, islets completely separated from their normally rich blood supply and innervation has been shown to survive and secrete insulin inside such devices (285). The most widely used immunobarrier approach is microencapsulation where islets are contained within a bead of alginate gel and then coat the bead with poly-L-lysine or some other material (such as agar) to provide permselectivity and strength (Figure 1-3; 286-291). Interest in the potential of microencapsulation has been enhanced by a recent report in which monkeys with spontaneous diabetes given adult porcine islets contained in alginate/polylysine capsules were cured for periods as long as 803 days without immunosuppression (287). This striking finding must be comfirmed by other investigators before this approach can be used in future human trials.

In spite of this apparent success, there are still major questions about how best to design microcapsules; some of the issues that must be taken into account include biocompatibility of material with host tissue and the contained islets, amount of permselectivity required, and distance from the surface of the capsule to the islet, which not only will influence delivery of oxygen and nutrients, but may also determine susceptibility to toxic cytokines such as interferon-γ and TNF-α. There are other concerns, such as release of antigens and debris of the capsular material in the peritoneal cavity, which may evoke an inflammatory response that might be difficult to stop because the capsules cannot be easily retrieved.

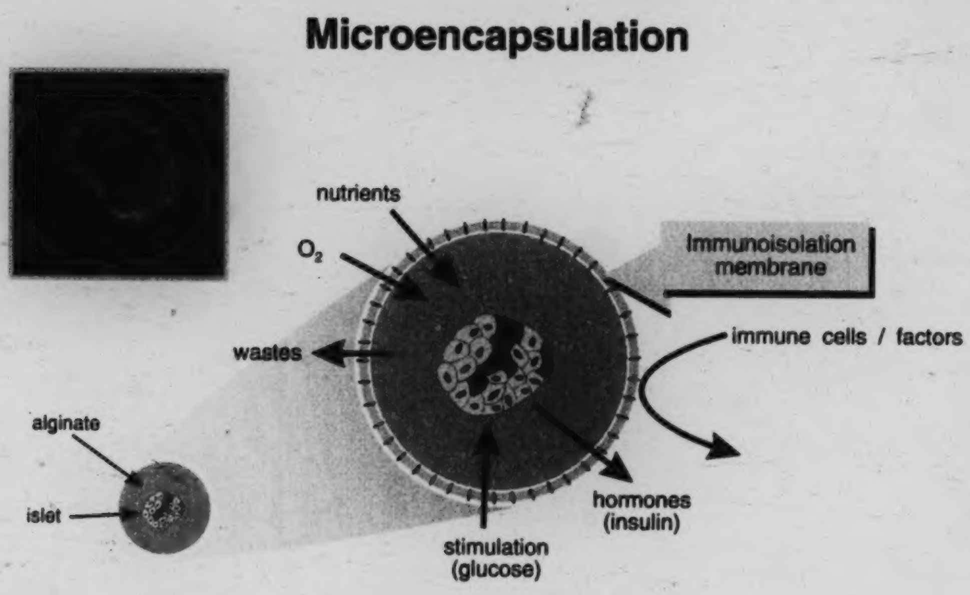
Microencapsulation
nutrients
O_2
Immunoisolation membrane
wastes
immune cells / factors
alginate
islet
hormones (insulin)
stimulation (glucose)

Figure 1-3. Schematic diagram of microencapsulated islet. The immunoisolation membrane protects the islet from immune cells yet allowing nutrients, glucose and oxygen to enter the membrane and cellular wastes, and hormones to get out of the membrane.

I-E) PANCREATIC B CELL GROWTH, DEVELOPMENT, AND REGENERATION

During embryogenesis, cells make developmental choices that commit themselves and their progeny to specific differentiation pathways (292). In mammals, the pancreas develops by fusion of dorsal and ventral primordia that appear as evaginations of the gut. This occurs in embryos of about 20 somites, which corresponds to day 10 of development in mouse and day 11 in the rat (292). The two primitive glands grow independently, forming both endocrine and exocrine tissue, and finally merge during midgestation (293, 294). The exocrine pancreas regulates electrolyte balance and secretes enzymes necessary for nutrient digestion and absorption. The endocrine pancreas, consisting of circumscribed collections of hormone-producing cells known as the Islets of Langerhans, senses and integrates nutrient signals in order to maintain euglycemia (294). Mature pancreatic islets are composed of four major cell types with distinct hormonal production profiles: the glucagon-secreting α cell, the insulin-secreting β cell, the somatostatin-secreting δ cell, and the pancreatic polypeptide-secreting F cell (292). The ontogeny of islet cells has been extensively studied. It is generally believed that all four types of endocrine cells arise from common multipotent precursor cells that bud off from the pancreatic ducts (295-297). Quantitative analyses of cell proliferation have demonstrated that cell division within the islet is insufficient to cause the observed growth of the islet tissue in fetal pancreas, indicating that there must exist a pool of rapidly proliferating precursor cells (298). The existence of such precursor cells in the ductal epithelium is supported by the morphological observation that fetal and neonatal islets are often closely associated with ductal epithelium (299, 300). One of the markers used to identify islet precursor cells is the enzyme catecholamine tyrosine hydroxylase which is expressed by immature α cells and by some cells in pancreatic ducts (301, 302). However, the relationship between cells expressing this marker during embryogenesis and the ductal epithelial cells is not clear. Another marker used is the intermediate filaments (IF) expression. The expression of intermediate filaments has been shown to be tightly regulated during differentiation and development (303, 304). Cytokeratins (CK), are the major IF proteins of epithelial cells and are expressed in a tissue-type specific manner, distinct subsets being expressed in different epithelial tissues (304, 305). Thus, CK expression has also been used to study epithelial cell differentiation and to determine the origin of certain epithelial tissues. In human pancreas, CKs 7 and 19 have been identified as the typical IF components of ductal epithelium, whereas CKs 8 and 18 are expressed in exocrine acini, ducts, and islets (306-308).

During islet development, precursor islet cells develop into one of the four cell types after passing through a transient phase in which two or more of the hormone genes are co-expressed in the same cell (309-312). Cells co-expressing glucagon and insulin first become evident in the pancreatic primordia at 9.5 days postcoitum (dpc) in mice, but their number decreases with further development. Somatostatin-secreting cells first appear at 14.5 dpc, and pancreatic polypeptide-producing cells appear around birth (311, 312). The molecular mechanisms underlying islet cell differentiation are still poorly understood, and what drives islet cells to become β cells is a topic of intense research. It is generally believed that cell-specific expression is regulated at the transcription level by transacting proteins which bind to short DNA sequences located upstream from the transcription initiation site (292). Recently, several genes within the homeobox family of developmental regulators have been shown to be expressed in the early forming pancreas, including pancreatic and duodenal homeobox gene-1 (PDX-1), Pax4, Pax6 and ISL-1 (313). PDX-1 was independently discovered by multiple laboratories (314-316) and has been described by a variety of names: IDX-1 (316), IPF-1 (315), STF-1 (314), IUF-1 (317). PDX-1 appears to be a "master regulator" of pancreas development and β cell differentiation and function (318). In murine gene inactivation models and in a human subject with a homozygous mutation of the PDX-1 gene, the pancreas fails to develop. In the adult endocrine pancreas, PDX-1 is primarily expressed in β cells, where it is a key factor in the upregulation of insulin gene transcription and appears to have a role in the regulation of the somatostatin, glucokinase, glucose transporter-2, and islet amyloid polypeptide genes (318). Recent studies also suggest a role for PDX-1 in the neogenesis and proliferation of β cells (319). The β cell in the adult endocrine pancreas has a life span of approximately 30 days, after which it undergoes programmed cell death (apoptosis; 320). The senescent β cells are replaced by processes of replication (proliferation) and by the neogenesis of new β cells derived from progenitor cells that bud from the ducts of the exocrine pancreas (mentioned above). Thus the entire mass of β cells in the endocrine pancreas turns over every 30 days and, at any given time, is dependent upon the balance among the relative rates of neogenesis, proliferation, and apoptosis (320). This circumstance is an important aspect for the maintenance of glucose homeostasis because the ability of the pancreas to produce sufficient insulin to meet the body's needs depends on the appropriate size of the β-cell mass. A reduction in β-cell mass results in an insufficiency in the production of insulin, causing diabetes mellitus. In IDDM, the β-cell mass is markedly reduced, owing to extensive immune destruction. Islet-1 (ISL-1) is required for the formation of dorsal mesenchyme, and thereafter for the generation of dorsal islet cells (321), Pax4 and Pax6 for the differentiation of β cells (322) and α cells (323), respectively, and BETA2/NeuroD for the development of both endocrine and exocrine cells (324).

I-F) OBJECTIVES AND GENERAL OUTLINE

The main objective of this thesis is to characterize the cells present in NPI as well as determine whether they will be susceptible to humoral and/or cell-mediated destruction induced by human immune cells. Several studies have demonstrated that Galα(1,3)Gal expression on porcine islets is confined to intra-islet capillary endothelial cells (196, 198) which may be removed during the isolation procedure. Thus, it has been speculated that porcine islets may not be hyperacutely rejected when transplanted into humans. However, it has been shown that fetal porcine islet cells were sensitive to human antibody/complement-mediated lysis *in vitro* (325) and rapid destruction of an isolated islet graft has also been described in rabbit-to-primate *in vivo* model (326). The expression of Galα(1,3)Gal on NPI and the sensitivity of these tissues to humoral-mediated destruction is not known. If NPI will be used to treat patients with type I diabetes in the future, it is critical that we must first examine whether these tissues will be susceptible to human antibody and complement-mediated lysis.

It has been the general consensus that porcine islet endocrine cells (such as insulin-producing β cells) do not express Galα(1,3)Gal. Previous histological studies demonstrated that adult, fresh neonatal and fetal porcine islet cells (apart from endothelial cells) lack Galα(1,3)Gal (209). In addition, double immunostaining studies showed that Gal-positive cells do not secrete insulin, glucagon, or somatostatin and these may be spared from potential antibody/complement-mediated rejection after transplantation. However, studies presented in Chapter II of this text demonstrated that insulin- or glucagon-secreting cells in NPI also express Galα(1,3)Gal and both Gal-positive and Gal-negative cells are susceptible to lysis mediated by human antibody and/or complement *in vitro*. The reason for this controversy is not clear. Since majority of these studies used adult pigs we suspect the Galα(1,3)Gal expression on porcine islets may be age-dependent. Thus, in Chapter III the expression of Galα(1,3)Gal on non-matured, *in vitro* and *in vivo* matured NPI as well as adult pig islets was further examined.

In Chapter II, NPI was shown to be susceptible to lysis induced by human antibody and complement *in vitro*. A number of strategies to protect islets from destruction by immune cells has been suggested. One of these strategies is to put the islets inside a protective membrane such as microcapsules. In Chapter IV, the efficacy of microencapsulation in protecting NPI from the cytolytic effects of human antibody and complement *in vitro* was assessed. In addition, the capacity of microencapsulated NPI to reverse hyperglycemia in diabetic nude mice was also examined. Finally, the other immunological hurdle that might limit the transplantation of NPI into humans is the cell-mediated immune response. In chapter V, the susceptibility of NPI to cell-mediated injury *in vitro* was determined.

Overall, the studies presented in this manuscript deal with two very important issues that will limit the use of NPI for transplantation into patients with type 1 diabetes. First, the shortage in human islet supply might be possibly be overcome by the use of NPI. These islets as has been demonstrated by Korbutt *et al*. (162) are easily maintained in tissue culture and have the potential to grow (as supported in Chapter III and IV). The growth potential of NPI is an advantage because it offers the possibility of further understanding how islet cells develop and mature into insulin-producing β cells. Thereby, help investigators in diabetes research design methods in enhancing growth and possibly create cells (by tissue engineering) that will be similar to β cells in function but lack antigens recognized by immune cells. In addition, the possibility of co-transplanting adult pig islets with NPI is a very attractive proposition. In this situation, the time that NPI requires to grow and secrete sufficient insulin to maintain normoglycemia will not be a limitation since adult pig islets will sustain normoglycemia during early weeks post-transplantation and NPI will maintain euglycemia long-term. The second important issue is the immunological responses to NPI (presented in Chapter II and Chapter V). This issue demands intensive research so investigators in this field will have better understanding of the immune cells involve in destruction of NPI grafts thereby help them design methods or treatments to prevent rejection (as discussed in Chapter IV). The knowledge acquired in these studies will also help clinicians decide what type of treatment a patient will need to keep the islet graft.

I-G) REFERENCES

1. Jackson N. Diabetes mellitus information. South African Diabetes Association. WEB Solutions 1997; 1. (www.websol.co.za/webmed/diabetes.html).

2. Canadian Diabetes Association and Statistics Canada. Diabetes in Canada. Diabetes Resources Network 1999; 1-5. (www.mnsi.net/~domarico/service1.html).

3. World Health Organization. Report of the WHO Expert Committee on Diabetes Mellitus. Geneva: WHO. 1985; 727: 1-113.

4. Rewers M. The changing face of the epidemiology of insulin-dependent diabetes mellitus: Research designs and models of disease causation. Ann Med. 1991; 23: 419-426.

5. Rewers M, LaPorte RE, King H, Tuomilehto J. Trends in the prevalence and incidence of diabetes: Insulin-dependent diabetes mellitus in childhood. World Health Stat Q 1988; 41: 179-189.

6. The Diabetes Control and Complications Trial (DCCT) Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993; 329: 977-986.

7. Bennett PH, Rewers MJ, Knowler WC. Epidemiology of diabetes mellitus. In: Porte D, Sherwin RS, eds. Ellenberg and Rifkin's Diabetes Mellitus 5th edn., Connecticut: Appleton and Lange, 1997: 373-400.

8. Atkinson MA, Maclaren NK. Islet cell autoantigens in insulin-dependent diabetes. J Clin Invest 1993; 92: 1608-1616.

9. Botazzo GF, Florin-Christensen A, Doniach S. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 1974; 2: 1279-1283.

10. Palmer JP, Asplin CM, Clemons P, *et al.* Insulin antibodies in insulin-dependent diabetes before insulin treatment. Science 1983; 222: 1337-1339.

11. Vardi P, Dib SA, Tuttleman M, *et al.* Competitive insulin autoantibody assay: Prospective evaluation of subjects at high risk for development of type I diabetes mellitus. Diabetes 1987; 36: 1286-1291.

12. Baekkeskov S, Nielsen JH, Marner B, *et al.* Autoantibodies in newly-diagnosed diabetic children immunoprecipitate specific human pancreatic cell proteins. Nature 1982; 298: 167-169.

13. Baekkeskov S, Aanstoot H, Christgau S, *et al.* Identification of the 64k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990; 347: 151-156.

14. Pietropaolo M, Castano L, Babu S, *et al.* Islet cell autoantigen 69 kD (ICA69). J Clin Invest 1993; 92: 359-371.

15. Christie MR, Hollands JA, Brown TJ, *et al.* Detection of pancreatic islet 64,000 M1 autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. J Clin Invest 1993; 92: 240-248.

16. Rabin DU, Pleasic SM, Shapiro JA, *et al.* Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. J Immunol 1994; 152: 3183-3187.

17. Erlich H, Griffith RL, Bugawan TL, *et al.* Implication of specific DQB1 alleles in genetic susceptibility and resistance by identification of IDDM siblings with novel HLABQB1 allele and unusual DR2 and DR1 haplotypes. Diabetes 1991; 40: 478-481.

18. Pugliese A, Moromisato R, Awdeh ZL, *et al.* HLA-DQB1*0602 confers dominant protection from IDDM even among ICA positive first degree relatives. Presented at the 13th International Immunology and Diabetes Workshop, Montvillargenne, France. 1994.

19. Chase HP, Voss MA, Butler-Simon N, *et al.* Diagnosis of pre-type 1 diabetes. J Pediatr 1987; 111: 807-812.

20. MacLaren N, Horne G, Spillar R, *et al.* The feasibility of using ICA to predict IDDM in U.S. school children. Diabetes 1990; 39: 122A.

21. Levy-Marchal C, Tichet J, Fajardy I, *et al.* Follow-up of children from a background population with high ICA titres. Diabetologia 1992; 35: A32.

22. Owerbach D, Gabbay KH. Localization of a type 1 diabetes susceptibility locus to the variable tandem repeat region flanking the insulin gene. Diabetes 1993; 42: 1708-1714.

23. Davies JL, Kawaguchi Y, Bennett ST, *et al.* A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994; 371: 130-136.

24. Hashimoto L, Habita C, Beressi JP, *et al.* Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q. Nature 1994; 371: 161-164.

25. Owerbach D, Gabbay KH. The HOXD8 locus (2q31) is linked to type 1 diabetes: Interaction with chromosomes 6 and 11 disease susceptibility genes. Diabetes 1995; 44: 132-136.

26. Field LL, Tobias R, Magnus T. A locus on chromosome 15q26 (IDDM3) produces susceptibility to insulin-dependent diabetes mellitus. Nature Genet 1994; 8: 189-194.

27. Maugh TH. Virus isolated from juvenile diabetic. Science 1979; 204: 1187.

28. Helmke K, Otten A, Willems W, *et al.* Islet cell antibodies and the development of diabetes mellitus in relation to mumps infection and mumps vaccination. Diabetologia 1986; 29: 30-33.

29. Ginsberg-Fellner F, Witt ME, Fedun B, *et al.* Diabetes mellitus and autoimmunity in patients with congenital rubella syndrome. Rev Infect Dis 1985; 7 (Suppl 1): S170-175.

30. Rayfield EJ, Seto Y. Viruses and pathogenesis of diabetes mellitus. Diabetes 1978; 27: 1126-1142.

31. Gamble DR, Taylor KW. Seasonal incidence of diabetes mellitus. Br Med J 1969; 3: 631-633.

32. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 1965; 14: 619-633.

33. Bodansky HG, Grant PJ, Dean BM, *et al.* Islet-cell antibodies and insulin autoantibodies in association with common viral infections. Lancet 1986; 2: 1351-1353.

34. Champsaur HF, Bottazzo GF, Bertrams J, *et al.* Virologic, immunologic, and genetic factors in insulin-dependent diabetes mellitus. J Pediatr 1982; 100: 15-20.

35. Uriarte A, Cabrera E, Ventura R, *et al.* Islet cell antibodies and ECHO-4 virus infection. Diabetologia 1987; 30: 590A.

36. Ginsberg-Fellner F, Witt ME, Yagihashi S, *et al.* Congenital rubella syndrome as a model for type 1 (insulin-dependent) diabetes mellitus: Increased prevalence of islet cell surface antibodies. Diabetologia 1984; 27: 87-89.

37. Rewers M, Atkinson M. The possible role of enterovirus in diabetes mellitus. In: Rotbart H, ed. Human Enterovirus Infections. Washington DC: American Society for Microbiology, 1995: 353-385.

38. Kaufman DL, Erlander MG, Clare-Salzler M, *et al.* Autoimmunity to two forms of glutamate decarboxylase in insulin dependent diabetes mellitus. J Clin Invest 1992; 89: 283-292.

39. Scherbaum WA, Hampl W, Muir P, *et al.* No association between islet cell antibodies and cocksackie B, mumps, rubella, and cytomegalovirus antibodies in non-diabetic individuals aged 7-19 years. Diabetologia 1991; 34: 835-838.

40. Vialettes B, Demicco P, Badier C, et al. Anti-coxsackie B IgG and IgM antibodies in first degree relatives of type 1 (insulin-dependent) diabetic patients: Lack of association with islet cell antibodies. Diabetologia 1987; 30: 594A.

41. Hyoty H, Hiltunen M, Knip M, *et al.* A prospective study on the role of enterovirus infection in the pathogenesis of IDDM. Presented at the 13th International Immunology and Diabetes Workshop, Montvillargenne, France, 1994.

42. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979; 300: 1173-1179.

43. Champsaur H, Dussaiz E, Samolyk F, Fabre M, Bach C, Assan R. Diabetes and Coxsackie virus B5 infection. Lancet 1980; 1: 251.

44. King ML, Shaikh A, Bidwell D, *et al.* Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. Lancet 1983; 1: 1397-1399.

45. Banatvala JE, Schernthaner G, Schober E, *et al.* Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. Lancet 1985; 1: 1409-1412.

46. Jenson AB, Rosenberg HS, Notkins AL. Pancreatic islet-cell damage in children with fatal viral infections. Lancet 1980; 2: 354-358.

47. Champsaur H, Bottazzo G, Bertrams J, *et al.* Virologic, immunologic, and genetic factors in insulin-dependent diabetes mellitus. J Pediat 1982: 100: 15-20.

48. Atkinson MA, Winter WE, Skordis N, *et al.* Dietary protein restriction reduces the frequency and delays the onset of insulin dependent diabetes in BB rats. Autoimmunity 1988; 2: 11-20.

49. Martin JM, Trink B, Daneman D, *et al.* Milk proteins in the etiology of insulin-dependent diabetes mellitus (IDDM). Ann Med 1991; 23: 447-452.

50. Pietropaolo M, Eisenbarth GS. Biochemical determination of antibodies to three recombinant human autoantigens: High predictive value and stability of patterns. Diabetes 1994; 43: 153A.

51. Atkinson MA, Bowman MA, Kao KJ, *et al.* Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. N Engl J Med 1993; 329: 1853-1858.

52. Norris JM, Beaty B, Eisenbarth GS, *et al.* Infant diet and prediabetic autoimmunity. The Diabetes Autoimmunity Study in the Young. Presented at the 13th International Immunology and Diabetes Workshop, Montvillargenne, France, 1994.

53. Fuechtenbusch M, Ziegler AG, Shibuya N, *et al.* Prevalence of BSA antibodies in IDDM first degree relatives. Presented at the 13th International Immunology and Diabetes Workshop, Montvillargene, France, 1994.

54. Krokowski M, Caillat-Zuckman S, Timsit J, *et al.* Anti-bovine serum albumin antibodies: Genetic heterogeneity and clinical relevance in adult-onset IDDM. Diabetes Care 1995; 18: 170-173.

55. Elias D, Prigozin H, Polak N, *et al.* Autoimmune diabetes induced by the B-cell toxin STZ. Immunity to 60-kDa heat shock protein and to insulin. Diabetes 1994; 43: 992-998.

56. Rayfield EJ, Ishimura K. Environmental factors and insulin dependent diabetes mellitus. Diabet Metab Rev 1987; 3: 925-957.

57. Toniolo A, Onodera T, Yoon JW, Notkins AL. Induction of diabetes by cumulative environmental insults from viruses and chemicals. Nature 1980; 288: 383-385.

58. Kolb H, Pozzilli P. Cow's milk and type I diabetes: the gut immune system deserves attention. Immunol Today 1999; 20: 108-110.

59. Elliott RB, Bibby N, Reddy S. In: Laron Z, Kerp M eds. Genetic and environmental risk factors for type I diabetes (IDDM). Freund Publishing House 1997: 57-69.

60. Banting FG, Best CH. The internal secretion of the pancreas. J Lab Clin Med 1922; 7: 256-271.

61. Purello F, Pipeleers D. Transplantation in diabetes: A cell biological problem. J Endocrinol Invest 1995; 18: 311-319.

62. Von Mering J, Minkowski O. Diabetes mellitus after pancreas extirpation. *Archiv fur Experimentale Pathologie und Pharmacologie* 1889; 26: 111-127.

63. Williams PW. Notes on diabetes treated with extract and by grafts of sheep pancreas. Br Med J 1894; 2: 1303.

64. Browning H, Rasnick P. Homologous and heterologous transplantation of pancreatic tissue in normal and diabetic mice. Yale J Biol Med 1951; 24: 141-152.

65. Brooks JR, Gifford GH. Pancreatic homotransplantation. Transplant Bull 1959; 6: 100-103.

66. Brooks JR. Endocrine tissue transplantation. Springfield: Charles C. Thomas, 1962: 83-85.

67. Kelly WD, Lillehei RC, Merkel FK. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. Surgery 1967; 61: 827-835.

68. Lillehei RC, Simmons RL, Najarian JS, *et al.* Pancreatico-duodenal allotransplantation: experimental and clinical experience. Ann Surg 1970; 172: 405-436.

69. Lillehei RC, Ruiz JO, Aquino C, Goetz FC. Transplantation of the pancreas. Acta Endocrinol (Copenh) 1976; 83 (Suppl 205): 303-320.

70. Sutherland DER, Goetz FC, Carpenter AM, Najarian JS, Lillehei RC. Pancreaticoduodenal grafts: clinical and pathological observations in uremic versus nonuremic recipients. Oxford: Excerpta Medica, 1979: 190-195.

71. Sutherland DER, Gruessner A. Pancreas transplantation in the United States as reported to the United Network for Organ Sharing (UNOS) and analyzed by the International Pancreas Transplant Registry. In: Terasaki and Cecka, eds. Clinical transplants 1995. Los Angeles: UCLA Tissue Typing Laboratory 1995: 49-67.

72. Calafiore R. Perspectives in pancreatic and islet cell transplantation for the therapy of IDDM. Diabetes Care 1997; 20: 889-896.

73. Gruessner RWG, Burke GW, Stratta R, *et al.* A multicenter analysis of the first experience with FK506 for induction and rescue therapy after pancreas transplantation. Transplantation 1996; 61: 261-273.

74. Gruessner A, Sutherland DER. Pancreas transplants for United States and non-US cases as reported to the International Pancreas Transplant Registry and to the United Network for Organ Sharing. In Cecka JM, Terasaki PI, eds. Clinical Transplants 1997. Los Angeles: UCLA Typing Laboratory, 1998: 45-59.

75. Sutherland DER. Pancreas transplants. Br J Surg 1994; 81: 2-4.

76. Purello F, Pipeleers D. Transplantation in diabetes: a cell biological problem. J Endocrinol Invest 1995; 18: 311-319.

77. Penn I. Cancers following cyclosporin therapy. Transplantation 1987; 43; 32.

78. Freuten G, Mihatsch MJ. Risk factors for cyclosporine-induced nephropathy in patients with autoimmune disease. N Engl J Med 1992; 326: 1654.

79. Sibley RK, Sutherland DER, Goetz F, Michael H. Recurrent diabetes mellitus in the pancreas iso- and allograft: a light and electron microscopy immunohistochemical analysis of four cases. Lab Invest 1985; 53: 132-144.

80. Tyden G, Reinholt FP, Sundkvist G, Bolinder J. Recurrence of autoimmune diabetes mellitus in recipients of cadaveric pancreatic grafts. N Engl J Med 1996; 335: 860-863.

81. Eisenbarth GS, Stegall M. Islet and pancreatic transplantation: Autoimmunity and alloimmunity. N Engl J Med 1996; 335: 888-889.

82. Rajotte RV, Steward HL, Voss WAG, *et al.* Viability studies on frozen-thawed rat islets of Langerhans. Cryobiology 1977; 14: 116.

83. Bretzel RG, Beulle B, Schaefer S, *et al.* Cryopreservation and tissue culture of pancreatic islets for transplantation in experimental diabetes mellitus. Diabetes 1979; 25: 377.

84. Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabetes mellitus - from bench to bedside. Exp Clin Endocrinol 1995; 103: 143-159.

85. Langerhans P. *Beitrage zur mikroskopischen anatomie der bauchspeicheldruse. Inauguraldissertation, Medizinische Fakultat, Friedrich-Wilhelm-Universitat Berlin,* Lange, Berlin, 1869.

86. Minkowski O. *Weitere Mittheilungen uber den diabetes mellitus nach extirpation des pankreas. Berl Klin Wochenschr* 1892; 29: 90-93.

87. Van Beek C. Leonid V. Sobolev 1876-1919. Diabetes 1958; 7: 245-248.

88. Hellerstrom C. A method for the microdissection of intact pancreatic islets of mammals. Acta Endocrinol 1964; 45: 122-132.

89. Moskalewski S. Isolation and culture of the islets of Langerhans of the guinea pig. Gen Comp Endocrinol 1965; 5: 342-353.

90. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 1967; 16: 35-39.

91. Lindall AW, Steffes MW, Sorensen R. Immunoassayable content of subcellular fractions of rat islets. Endocrinilogy 1969; 85: 218-223.

92. Scharp DW, Kemp CB, Knight MJ, *et al.* The use of ficoll in the preparation of viable islets of Langerhans from the rat pancreas. Transplantation 1973; 16: 686-689.

93. Younoszai R, Sorensen RL, Lindall AW. Homotransplantation of isolated pancreatic islets. Diabetes (Suppl 1), 406 (Abstract).

94. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islet in rats. Surgery 1972; 72: 175-186.

95. Reckard C, Barker C. Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers. Transplant Proc 1973; 5: 761-763.

96. Kemp CB, Knight MJ, Scharp DW, *et al.* Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. Diabetologia 1973; 9: 486-491.

97. Matas AJ, Payne WD, Grotting JC, *et al.* Portal versus systemic transplantation of dispersed neonatal pancreas. Transplantation 1977; 24: 333-337.

98. Brown J, Mullen Y, Clark W, *et al.* Importance of hepatic portal circulation for insulin action in streptozotocin-diabetic rats transplanted with foetal pancreas. J Clin Invest 1979; 64: 1688-1694.

99. Reece-Smith H, McShane P, Morris PJ. Glucose and insulin changes following a renoportal shunt in streptozotocin diabetic rats with pancreatic islet isografts under the kidney capsule. Diabetologia 1982; 23: 343-346.

100. Cuthbertson RA, Mandel TE. A comparison of portal versus systemic venous drainage in murine foetal pancreatic islet transplantation. Aust J Exp Biol Med Sci 1986; 64: 175-184.

101. Lazarow A, Wells LJ, Carpenter AM, Hegre OD. Islet differentiation, organ culture and transplantation. Diabetes 1973; 22: 413-426.

102. Panijayanaud P, Soroff HS, Monaco AP. Pancreatic islet isografts in mice. Surg Forum 1973; 24: 329-331.

103. Lorenz D, Peterman J, Beckert R, *et al.* Transplantation of isologous islets of Langerhans in diabetic rats. Acta Diabetol Lat 1975; 12: 30-40.

104. Rumpf KD, Lohlein D, Pichlmayr R. Multiple transplantations of islets of Langerhans. Eur Surg Res 1977; 9: 403-410.

105. Henriksson C, Bergmark J, Claes G. Metabolic response to isologous transplantation of small numbers of isolated islets of Langerhans in the rat. Eur Surg Res 1977; 9: 411-418.

106. Finch DRA, Wise PH, Morris PJ. Successful intrasplenic transplantation of syngeneic and allogeneic isolated pancreatic islets. Diabetologia 1977; 13: 195-199.

107. Reckard CR, Franklin W, Schulak JA. Intrasplenic vs intraportal pancreatic islet transplantation. Quantitative, qualitative and immunologic aspects. Trans Am Soc Artif Organs 1978; 24: 232-234.

108. Yasunami Y, Lacy PE, Finke E. A new site for islet transplantation - a peritoneal omental pouch. Transplantation 1983; 36: 181-182.

109. Reece-Smith H, Du Toit DF, McShane P, Morris PJ. Prolonged survival of pancreatic islet allografts transplanted beneath the renal capsule. Transplantation 1981; 31: 301-306.

110. Serie J, Hickey GE, Schmitt RV, Hegre OD. Prolongation of cultured isolated neonatal islet xenografts without immunosuppression. Transplantation 1983; 36: 6-11.

111. Gray DWR, Reece-Smith H, Fairbrother B, *et al.* Isolated pancreatic islet allografts in rats rendered immunologically unresponsive to renal allografts. The effect of the site of transplantation. Transplantation 1984; 37: 434-437.

112. Bobzien B, Yasunami Y, Majercik M, *et al.* Intratesticular transplants of islet xenografts (rat to mouse). Diabetes 1983; 32: 213-216.

113. Tze WJ, Tai J. Successful intracerebral allotransplantation of purified pancreatic endocrine cells in diabetic rats. Diabetes 1983; 32: 1185-1187.

114. Posselt AM, Barker CF, Tomaszewski JE, *et al.* Induction of donor-specific unresponsiveness by intrathymic islet transplantation. Science 1990; 249: 1293-1295.

115. Scharp DW. Clinical feasibility of islet transplantation. Transplant Proc 1984; 16: 820-825.

116. Mirkovitch V, Campiche M. Intrasplenic autotransplantation of canine pancreatic tissue: Maintenance of normoglycemia after total pancreatectomy. Eur Surg Res 1977; 9: 173-190.

117. Kretschmer GJ, Sutherland DER, Matas AF, *et al.* The dispersed pancreas: Transplantation without islet purification in totally pancreatectomized dogs. Diabetologia 1977; 13: 495-502.

118. Sutherland DER, Steffes MW, Bauer GE, *et al.* Isolation of human and porcine islets of Langerhans and islet transplantation in pigs. J Surg Res 1974; 16: 102-111.

119. Scharp DW, Murphy JJ, Newton WT, *et al.* Transplantation of islets of Langerhans in diabetic rhesus monkeys. Surgery 1975; 88: 100-105.

120. Scharp DW, Downing R, Merrel RC, Greider M. Isolating the elusive islet. Diabetes 1980; 29: 19-30.

121. Kolb E, Ruckert R, Largardier F. Intraportal and intrasplenic autotransplantation of pancreatic islets in the dog. Eur Surg Res 1977; 9: 419-426.

122. Schulak JA, Stuart FP, Reckard CR. Physiologic aspects of intrasplenic autotransplantation of pancreatic fragments in the dog after 24 hours of cold storage. J Surg Res 1978; 24: 125-131.

123. Mehigan DG, Zuidema GD, Cameron JL. Pancreatic islet transplantation in dogs: Critical factors in technique. Am J Surg 1981; 141: 208-212.

124. Horaguchi A, Merrel RC. Preparation of viable islet cells from dogs by a new method. Diabetes 1981; 30: 455-458.

125. Warnock GL, Rajotte RV, Procyshyn AW. Normoglycemia after reflux of islet containing fragments into the splenic vascular bed in dogs. Diabetes 1983; 32: 452-458.

126. Alderson D, Farndon JR. The metabolic effects of islet transplantation in the diabetic dog. Transplant Proc 1984; 16: 831-833.

127. Hering BJ, Ricordi C. Results, research priorities, and reasons for optimism: Islet transplantation for patients with type 1 diabetes. Graft 1999; 2: 12-27.

128. Brennan DC, Flavin KS, Scharp DW, *et al.* Long-term (>30 months) follow-up of a single successful kedney-islet transplant recipient. Transplant Proc 1997; 29: 2231-2233.

129. Meyer C, Hering BJ, Grossman R, *et al.* Improved glucose counterregulation and autonomic symptoms after intraportal islet transplants alone in patients with long-standing type 1 diabetes mellitus. Transplantation 1998; 66: 233-240.

130. Leone JP, Kendall DM, Reinsmoen N, *et al.* Immediate insulin-independence after retransplantation of islets prepared from an allograft pancreatectomy in type 1 diabetic patient. Transplant Proc 1998; 30: 319.

131. Largiader F, Kolb E, Binswanger U. A long-term functioning human pancreatic islet allotransplant. Transplantation 1980; 29: 76-77.

132. Soon-Shiong P, Heintz RE, Merideth N, *et al.* Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. Lancet 1994; 343: 950-951.

133. Hauptman PJ, O'Connor KJ. Procurement and allocation of solid organs for transplantation. N Engl J Med 1997; 336: 422-431.

134. La Porte RE, Matsushima M, Chang Y-F. Prevalence and incidence of insulin-dependent diabetes. In: Harris MI, Cowie CC, Stern MP, Boyko EJ, Reiber GE, Bennett PH, eds. Diabetes in America. 2nd edn. Washington, DC: US Govt. Printing Office, 1995: 37-46 (NIH publ. No 95-1468).

135. Kendall DM, Sutherland DER, Najarian JS, *et al.* Effects of hemipancreatectomy on insulin secretion and glucose tolerance in healthy humans. N Engl J Med 1990; 322: 898-903.

136. Weir GC, Bonner-Weir S. Scientific and political impediments to successful islet transplantation. Diabetes 1997; 46: 1247-1256.

137. Parker W, Saadi S, Lin SS, *et al.* Transplantation of discordant xenografts: a challenge revisited. Immunol Today 1996; 17: 373-378.

138. Cooper DKC, Ye Y, Rolf LL Jr, Zuhdi N. The pig as a potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. Xenotransplantation: The transplantation of organs and tissues between species, 1st edn. New York: Springer-Verlag, 1991: 481-499.

139. Maki T, O'Neil JJ, Porter J, *et al.* Porcine islets for xenotransplantation. Transplantation 1996; 62: 136-138.

140. Ricordi C, Finke EH, Lacy PE. A method for the mass isolation of islets from the adult pig pancreas. Diabetes 1986; 35: 649-653.

141. Ricordi C, Lacy PE. Renal subcapsular xenotransplantation of purified porcine islets. Transplantation 1977; 44: 721-723.

142. Marchetti P, Zappella A, Giannarelli R, et al. Isolation of islets of Langerhans from adult pig pancreas. Transplant Proc 1988; 20: 707-708.

143. Hesse VJ, Weye J, Meyer G, Isselhard W, Pilchmaier H. Long-term results after porcine islet transplantation. Transplant Proc 1989; 21: 2763-2764.

144. Horaguchi A, Merrel RC. Preparation of viable islet cells from dogs by a new method. Diabetes 1981; 30: 455-458.

145. Ricordi C, Socci C, Davalli AM, *et al.* Isolation of the elusive pig islet. Surgery 1990; 107: 688-694.

146. Marchetti P, Finke EH, Gerasimidi-Vazeou A, *et al.* Automated large scale isolation, in vitro function and xenotransplantation of porcine islets of Langerhans. Transplantation 1991; 52: 209-213.

147. Deng S, Buhler L, Andereggen E, *et al.* Islet isolation from slaughterhouse pig pancreata: evidence of *in vitro* and *in vivo* function. Transplant Proc 1994; 26: 3396.

148. Basta G, Osticioli L, Tortoioli C, *et al.* Method for mass separation and morphologic and functional qualification of adult porcine pancreatic islets. Xenotransplantation 1995; 2: 120-122.

149. Warnock GL, Katyal D, Okamura J, *et al.* Studies of the isolation, viability, and preservation of purified islets after surgical pancreatectomy in large pigs. Xenotransplantation 1995; 2: 161-164.

150. Vantyghem MC, Kerr-Conte J, Pattou F, *et al.* Immunohistochemical and ultrastructural study of adult porcine endocrine pancreas during the different steps of islet isolation. Histochem Cell Biol 1996; 106: 511-519.

151. Hesse UJ, Danis J, Meyer G, *et al.* Preparation and transplantation of pancreatic islet tissue in Landrace pigs and in the Munich miniature swine troll. Transplant Proc 1990; 22: 793.

152. Brandhorst D, Hering BJ, Brandhorst H, *et al.* Dietary treatment with soybean oil improves porcine islet culture and reduces islet immunogenecity. Transplant Proc 1994; 26: 613.

153. Ulrichs K, Bosse M, Wacker HH, *et al.* Histologic analysis of the porcine pancreas to improve yield and integrity after collagenase digestion. Transplant Proc 1994; 26: 610-612.

154. Kirchhof N, Hering BJ, Geiss V, *et al.* Evidence for breed-dependent differences in porcine islet of Langerhans. Transplant Proc 1994; 26: 616-617.

155. Groth CG, Korsgren O, Tibell A, *et al.* Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344: 1402-1404.

156. Korsgren O, Jansson L, Eizirik D, Andersson A. Functional and morphological differentiation of fetal porcine islet-like clusters after transplantation into nude mice. Diabetologia 1991; 34: 379-386.

157. Lui X, Federlin KF, Bretzel RG, *et al.* Persistent reversal of diabetes by transplantation of fetal pig proislets into nude mice. Diabetes 1991; 40: 858-866.

158. Asplund K, Westman S, Hellerstrom C. Glucose stimulation of insulin secretion from the isolated pancreas of foetal and new born rats. Diabetologia 1969; 5: 260-262.

159. Asplund G. Dynamics of insulin release from the foetal and neonatal rat pancreas. Euro J Clin Invest 1973; 3: 338-344.

160. Rhoten WB. Insulin secretory dynamics during development of rat pancreas. Am J Physiol 1980; 239: E57-E63.

161. Hole RL, Pian-Smith MCM, Sherp GWG. Development of the biphasic response to glucose in fetal and neonatal rat pancreas. Am J Physiol 1988; 254: E167-E174.

162. Korbutt GS, Elliott JF, Ao Z, Smith D, *et al.* Large scale isolation, growth, and function of neonatal porcine islets. J Clin Invest 1996; 97: 2119-2129.

163. Schilling A, Land W, Pratschke E, *et al.* Dominant role of complement in the hyperacute xenograft rejection reaction. Surgery, Gynecology & Obstetrics 1976; 142: 29-32.

164. Auchincloss H, Jr. Xenogeneic transplantation: A review. Transplantation 1988; 46: 1-20.

165. Platt J, Vercellotti GM, Dalmasso AP, *et al.* Transplantation of discordant xenografts: A review of progress. Immunol Today 1990; 11: 450-456.

166. Platt JL, Bach FH. The barrier to xenotransplantation. Transplantation 1991; 52: 937-947.

167. Baldwin WM, III, Pruitt SK, Brauer RB, *et al.* Complement in organ transplantation: Contributions to inflammation, injury and rejection. Transplantation 1995; 59: 797-808.

168. Calne RY. Organ transplantation between widely disparate species. Transplant Proc 1970; 2: 550-556.

169. Platt JL, Vercellotti GM, Dalmasso AP, *et al.* Transplantation of discordant xenografts: A review of progress. Immunol Today 1990; 11: 450-456.

170. Perper RJ, Najarian JS. Experimental renal heterotransplantation. Transplantation 1966; 4: 377-388.

171. Hammer C, Suckfull M, Saumweber D. Evolutionary and immunological aspects of xenotransplantation. Transplant Proc 1992; 24: 2397-2400.

172. Zhao ZX, Termignon J-L, Cardoso J, *et al.* Hyperacute xenograft rejection in the swine-to-human donor-recipient combination. Transplantation 1994; 57: 245-249.

173. Vanhove B, de Martin R, Lipp J, Bach FH. Human xenoreactive natural antibodies of the IgM isotype activate pig endothelial cells. Xenotransplantation 1994; 1: 17-23.

174. Tuso PJ, Cramer DV, Yasunga C, *et al.* Removal of human xenoantibodies to pig vascular endothelium by perfusion of blood through pig kidneys and livers. Transplantation 1993; 55: 1375-1378.

175. Borche L, Thibaudeau K, Navenot JM, *et al.* Cytolytic effect of human anti-Gal IgM and complement on porcine endothelial cells: A kinetic analysis. Xenotransplantation 1994; 1: 125-131.

176. Koren E, Kujundzic M, Koscec M, *et al.* Cytotoxic effects of human preformed anti-GAL IgG and complement on cultured pig cells. Transplant Proc 1994; 26:1336-1339.

177. Saadi S, Platt JL. Transient perturbation of endothelial integrity induced by natural antibodies and complement. J Exp Med 1995; 181: 21-31.

178. Parker W, Bruno D, Holzknecht ZE, Platt JL. Characterization and affinity isolation of xenoreactive human natural antibodies. J Immunol 1994; 153: 3791-3803.

179. Kujundzic M, Koren E, Neethling FA, *et al.* Variability of anti-αGal antibodies in human serum and their relation to serum cytotoxicity against pig cells. Xenotransplantation 1994; 1: 58-65.

180. Galili U. Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: A major obstacle for xenotransplantation in humans. Immunol Today 1993; 14: 480-482.

181. Schaapherder AFM, Daha MR, Te Bulte M-T JW, *et al.* Antibody-dependent cell-mediated cytotoxicity against porcine endothelium induced by a majority of human sera. Transplantation 1994; 57: 1376-1382.

182. Inverardi L, Pardi R. Early events in cell-mediated recognition of vascularized xenografts: Cooperative interaction between selected lymphocyte subsets and natural antibodies. Immunol Rev 1994; 141: 71-931.

183. Watier H, Guillaumin J-M, Piller F, *et al.* Removal of terminal α-galactosyl residues from xenogeneic porcine endothelial cells: Decrease in complement-mediated cytotoxicity but persistence of IgG-mediated antibody-dependent cell-mediated cytotoxicity. Transplantation 1996; 62: 105-113.

184. Galili U, Macher BA, Buehler J, Shohet SB. Human natural anti-α-galactosyl IgG. II. The specific recognition of α(1→3)-linked galactose residues. J Exp Med 1985; 162: 573-582.

185. Galili U, Shohet SB, Kobrin E, *et al.* Man, apes, and old world monkeys differ from other mammals in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem 1988; 262: 17755-17762.

186. Galili U, Swanson K. Gene sequences suggest inactivation of α-1,3-galactosyltransferase in catarrhines after the divergence of apes from monkeys. Proc Natl Acad Sci USA 1988; 7401-7404.

187. Kaplon RJ, Michler RE, Xu H, *et al.* Absence of hyperacute rejection in newborn pig-to-baboon cardiac xenografts. Transplantation 1995; 59: 1-6.

188. Xu H, Edwards NM, Chen JM, *et al.* Newborn baboon serum lacks natural anti-pig xenoantibody. Transplantation 1995; 59: 1189-1194.

189. Michler RE, Xu H, O'Hair DP, *et al.* Newborn discordant cardiac xenotransplantation in primates: a model of natural antibody depletion. Transplant Proc 1996; 28: 651-652.

190. Itescu S, Kwiatkowski P, Artrip JH, *et al.* Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period. Hum Immunol 1998; 59: 275-286.

191. Galili U, Mandrell RE, Hamadeh RM, *et al.* Interaction between human natural anti-α-galactosyl immunoglobulin G and bacteria of the human flora. Infect Immun 1988; 56: 1730-1737.

192. Blakely ML, Van Der Werf WJ, Berndt MC, *et al.* Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection. Transplantation 1994; 58: 1059-1066.

193. Kwiatkowski P, Artrip JH, Edwards NM, *et al.* High-level porcine endothelial cell expression of α(1,2)-fucosyltransferase reduces human monocyte adhesion and activation. Transplantation 1999; 67: 219-226.

194. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC. Anti-pig IgM antibodies in human serum react predominantly with Galα(1,3)Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391-11395.

195. Oriol R, Ye Y, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433-1442.

196. McKenzie IFC, Xing PX, Vaughan HA, *et al.* Distribution of the major xenoantigen (gal(α1-3)gal) for pig to human xenografts. Transplant Immunol 1994; 2: 81-86.

197. Rydberg L, Groth CG, Moller E, *et al.* Is the Galα(1,3)Gal epitopes a major target for xenoantibodies on pig fetal islet cells? Xenotransplantation 1995; 2: 148-153.

198. McKenzie IFC, Koulmanda M, Mandel TE, *et al.* Pig-to-human xenotransplantation: the expression of Galα(1,3)Gal epitopes on pig islet cells. Xenotransplantation 1995; 2: 1-7.

199. Galili U. Antigal antibody prevents xenotransplantation. Science & Medicine 1998; 5: 28-37.

200. Cooper DKC, Good AH, Koren E, *et al.* Identification of α-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies: Relevance to discordant xenografting in man. Transplant Immunol 1993; 1: 198-205.

201. Neethling FA, Koren E, Ye Y, *et al.* Protection of pig kidney (PK15) cells from the cytotoxic effect of anti-pig antibodies by α-galactosyl oligosaccharides. Transplantation 1994; 57: 959-963.

202. Vaughan HA, Loveland BE, Sandrin MS. Galα(1,3)Gal is the major xenoepitope expressed on pig endothelial cells recognized by naturally occurring cytotoxic human antibodies. Transplantation 1994; 58: 879-882.

203. Collins BH, Cotterell AH, McCurry KR, *et al.* Cardiac xenografts between primate species provide evidence for the importance of the α-galactosyl determinant in hyperacute rejection. J Immunol 1995; 154: 5500-5510.

204. Platt JL, Holzknecht ZE. Porcine paletelet antigens recognized by human xenoreactive natural antibodies. Transplantation 1994; 57: 327-335.

205. Alvarado CG, Cotterell AH, McCurry KR, *et al.* Variation in the level of xenoantigen expression in porcine organs. Transplantation 1995; 59: 1589-1596.

206. McKenzie IFC, Koulmanda M, Sandrin MS, Mandel TE. Expression of galα(1,3)gal by porcine islet cells and its relevance to xenotransplantation. Xenotransplantation 1995; 2: 139-142.

207. Menger MD, Vajkoczy P, Beger C, Messmer K. Orientation of microvascular blood flow in pancreatic islet isografts. J Clin Invest 1994; 93: 2280-2285.

208. Korbutt GS, Aspeslet LJ, Rajotte RV, *et al.* Natural human antibody-mediated destruction of porcine neonatal islet cell grafts. Xenotransplantation 1996; 3: 207-216.

209. Bach FH, Auchincloss H, Jr., Robson SC. Xenotransplantation. In: Bach FH, Auchincloss H, Jr., eds. Transplantation Immunology. New York: Wiley-Liss, 1995: 305-338.

210. Bach FH, Winkler H, Ferran C, *et al.* Delayed xenograft rejection. Immunol Today 1996; 17: 379-384.

211. Lin Y, Vandeputte M, Waer M. Factors involved in rejection of concordant xenografts in complement-deficient rats. Transplantation 1997; 63: 1705-1712.

212. Fryer JP, Leventhal JR, Matas AJ. The emergence of xenotransplantation. Trant Immunol 1995; 3: 21-31.

213. Saadi S, Holznecht RA, Patte CP, *et al.* Complement-mediated regulation of tissue factor activity in endothelium. J Exp Med 1995; 182: 1807.

214. Lawson JH, Platt JL. Molecular barriers to xenotransplantation. Transplantation 1996; 62: 303-310.

215. Hofer E, Duchler M, Fuad SA, *et al.* Candidate natural killer cell receptors. Immunol Today 1992; 13: 429-430.

216. Inverardi L, Samaja M, Motterlini R. Early recognition of a discordant xenogeneic organ by human circulating lymphocytes. J Immunol 1992; 149: 1416-1423.

217. Kotasek D, Vercellotti GM, Ochoa AC, *et al*. Mechanism of cultured endothelial injury induced by lymphokine-activated killer cells. Cancer Res 1988; 48: 5528-5532.

218. Bach FH, Robson SC, Ferran C, et al. Xenotransplantation: Endothelial cell activation and beyond. Transplant Proc 1995; 27: 77-79.

219. Gill RG, Wolf L, Coulombe M. CD4+ T cells are both necessary and sufficient for islet xenograft rejection. Transplant Proc 1994; 26: 1203.

220. Wolf L, Coulombe M, Gill RG. Donor antigen-presenting cell-independent rejection of islet xenografts. Transplantation 1995; 1164-1170.

221. Marchetti P, Scharp DW, Finke EH, *et al.* Prolonged survival of discordant porcine islet xenografts. Transplantation 1996; 61: 1100-1102.

222. Pierson RN III, Winn HJ, Russell PS, Auchincloss H Jr. Xenogeneic skin graft rejection is especially dependent on CD4+ T cells. J Exp Med 1989; 170: 991-996.

223. Desai NM, Bassiri H, Odorico JS, *et al.* Pancreatic islet allograft and xenograft survival in CD8+ T-lymphocyte-deficient recipients. Transplant Proc 1993; 25: 961-964.

224. Osorio RW, Ascher NL, Stock PG. Prolongation of *in vivo* mouse islet allograft survival by modulation of MHC class I antigen. Transplantation 1994; 57: 783-788.

225. Murray AG, Khodadoust MM, Pober JS, Bothwell AL. Porcine aortic endothelial cells activate human T cells: Direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD28. Immunity 1994; 1: 57-63.

226. Yamada K, Sachs DH, Dersimonian H. Human anti-porcine xenogeneic T cell response: evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. J Immunol 1995; 155: 5249-5256.

227. Azimzadeh A, Anegon I, Thibaudeau K, *et al.* Removal of anti-Galα(1,3)Gal antibodies diminishes the cytotoxic effect of primate xenoreactive antibodies on rat endothelial cells. Transplant Proc 1997; 29: 2337.

228. Weiss RA. Transgenic pigs and virus adaptation. Nature 1998; 39: 327-328.

229. Pruitt SK, Baldwinn WM III, Marsh HC Jr, *et al.* The effect of soluble complement receptor type 1 on hyperacute xenograft rejection. Transplantation 1991; 52: 868-873.

230. Xia W, Fearon DT, Kirkman RL. Effect of repetitive doses of soluble human complement receptor type 1 on survival of discordant cardiac xenografts. Transplant Proc 1993; 25: 410-411.

231. Pruitt SK, Kirk AD, Bollinger RR, *et al.* The effect of soluble complement receptor type 1 on hyperacute rejection of porcine xenografts. Transplantation 1994; 57: 363-370.

232. Candinas D, Lesnikoski B-A, Robson SC, *et al.* Effect of repetitive high-dose treatment with soluble complement receptor type 1 and cobra venom factor on discordant xenograft survival. Transplantation 1996; 62: 336-342.

233. Chen C-G, Fisicaro N, Shinkel TA, *et al.* Reduction in Gal-α1,3-Gal epitope expression in transgenic mice expressing human H-transferase. Xenotransplantation 1996; 3: 69-75.

234. Sandrin MS, Fodor WL, Cohney S, *et al.* Reduction of the major porcine xenoantigen Galα(1,3)Gal by expression of α(1,2)fucosyltransferase. Xenotransplantation 1996; 3: 134-140.

235. Koike C, Kannagi R, Takuma Y, *et al.* Introduction of α(1,2)-fucosyltransferase and its effect on α-Gal epitopes in transgenic pig. Xenotransplantation 1996; 3: 81-86.

236. Sandrin MS, Fodor WL, Mouhtouris E, *et al.* Enzymatic remodelling of the carbohydrate surface of a xenogeneic cell substantially reduces human antibody binding and complement-mediated cytolysis. Nat Med 1995; 1: 1261-1267.

237. Osman N, McKenzie IFC, Mouhtouris E, Sandrin MS. Switching amino-terminal cytoplasmic domains of α(1,2)fucosyltransferase and α(1,3)galactosyltransferase alters the expression of H substance and Galα(1,3)Gal. J Biol Chem 1996; 271: 33105-33109.

238. Tearle RG, Tange MJ, Zannettino ZL, *et al.* The α-1,3-Galactosyltransferase knockout mouse. Transplantation 1996; 61: 13-19.

239. Tange MJ, Salvaris E, Romanella M, *et al.* Additive effects of CD59 expression in Gal knockout mice *in vitro* but not in an *ex vivo* model. Xenotransplantation. 1997; 4: 25-33.

240. McCurry KR, Kooyman DL, Alvarado CG, *et al.* Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. Nat Med 1995; 1: 423-427.

241. Cozzi E, White DJG. The generation of transgenic pigs as potential organ donors for humans. Nat Med 1995; 1: 964-966.

242. Cary N, Moody J, Yannoutsos N, *et al.* Tissue expression of human decay accelerating factor, a regulator of complement activation expressed in mice: A potential approach to inhibition of hyperacute xenograft rejection. Transplant Proc 1993; 25: 400-401.

243. Diamond L, Oldham ER, Platt JL, *et al.* Cell and tissue specific expression of a human CD59 minigene in transgenic mice. Transplant Proc 1994; 26: 1239.

244. Rosengard AM, Cary NR, Langford GA, *et al.* Tissue expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs-a potential approach for preventing xenograft rejection. Transplantation 1995; 59: 1325-1333.

245. Langford GA, Yannoutsos N, Cozzi E, *et al.* Production of pigs transgenic for human decay accelerating factor. Transplant Proc. 1995; 26: 1400-1401.

246. Lavitrano M, Stoppacciaro A, Bacci ML, *et al.* Human decay accelerating factor transgenic pigs for xenotransplantation obtained by sperm-mediated gene transfer. Transplant Proc 1999; 31: 972-974.

247. Pascher A, Poehlein C, Storck M, *et al.* Immunopathologic observations after xenogeneic liver perfusion using donor pigs transgenic for human decay-accelerating factor. Transplantation 1997; 64: 384-391.

248. Lenschow DJ, Zeng Y, Thistlethwaite JR, *et al.* Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. Science 1992; 257: 789-792.

249. Roy-Chaudhury P, Nickerson PW, Manfro RC, *et al.* CTLA4Ig attenuates accelerated rejection (presensitization) in the mouse islet allograft model. Transplantation 1997; 64: 172-175.

250. Weber CJ, Hagler MK, Chryssochoos JT, *et al.* CTLA4-Ig prolongs survival of microencapsulated neonatal porcine islet xenografts in diabetic NOD mice. Cell Transplant 1997; 6: 505-508.

251. Umezawa H, Ishizuka M, Takeuchi T, *et al.* Suppression of tissue graft rejection by spergualin. J Antibiot (Tokyo) 1985; 38: 283-284.

252. Kino T, Hatanaka H, Hashimoto M. A novel immunosuppressant isolated from a streptomyces. I. Fermentation, isolation, and physico-chemical biological characteristics. J Antibiotics 1987; 40: 1249-1255.

253. Morris RE, Hoyt G. Prolongation of rat heart allograft survival by RS-61443. Surg Forum 1989; 40: 337-338.

254. Morris RE, Meiser BM. Identification of a new pharmacologic action of an old compound. Med Sci Res 1989; 17: 609-610.

255. Kuchle CC, Thoenes GH, Langer KH. Prevention of kidney and skin graft rejection in rats by leflunomide, a new immunomodulating agent. Transplant Proc 1991; 23: 1083-1086.

256. Cramer DV, Chapman FA, Makowka L. The use of brequinar sodium for transplantation. Ann N Y Acad Sci 1993; 696: 216-226.

257. Gremlich S, Roduit R, Thorens B. Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic B cells. J Biol Chem 1997; 272: 3216-3222.

258. Murase N, Starzl TE, Demetris AJ, *et al.* Hamster-to-rat heart and liver xenotransplantation with FK506 plus antiproliferative drugs. Transplantation 1993; 55: 701-707.

259. McManus RP, O'Hair DP, Komorowski R, Scott JP. Immunosuppressant combinations in primate cardiac xenografts. A review. Ann N Y Acad Sci 1993; 696:281-284.

260. Wang M, Tu Y, Stepkowski SM, Kahan BD. Effect of cyclosporine alone or in combination with rapamycin and brequinar on survival of hamster heart xenograft in rats. Transplant Proc 1993; 25: 2876-2877.

261. Xiao F, Chong A, Foster P, *et al.* Effect of leflunomide in control of acute rejection in hamster-to-rat cardiac xenografts. Transplant Proc 1994; 26: 1263-1265.

262. Borel JF, Kis ZL. The discovery and development of cyclosporine (Sandimmune). Transplant Proc 1991; 23: 1867-1877.

263. Briggs JD. A critical review of immunosuppressive therapy. Immuno Lett 1991; 29: 89-95.

264. Propper DJ, Catto GRD. Immunological aspects of clinical renal transplantation. Immunol Lett 1991; 29: 65-68.

265. Baker CF, Billingham RE. Immunologically priveleged sites. Adv Immunol 1977; 25: 1-54.

266. Rayat GR, Korbutt GS, Elliott JF, Rajotte RV. Survival and function of syngeneic rat islet grafts placed within the thymus versus the kidney capsule. Cell Transplant 1997; 6: 597-602.

267. Selawry H, Whittington K. Extended survival of islets grafted into the intra-abdominally placed testis. Diabetes 1984; 33: 405-406.

268. Whitmore WF III, Karsh FL, Gittes RF. The role of germinal epithelium and spermatogenesis in the priveleged survival of intratesticular grafts. J Urol 1985; 134: 782-786.

269. Selawry HP, Whittington KB, Bellgrau D. Abdominal intratesticular islet xenograft survival in rats. Diabetes 1989; 220-223.

270. Selawry HP. Islet transplantation to immunopriveleged sites. In: Lanza RP, Chick WL, eds. Pancreatic Islet Transplantation. Pittsburgh, PA: RG Landes, 1994: 75-602.

271. Bellgrau D, Gold D, Selawry H, *et al.* A role of CD95 ligand in preventing graft rejection. Nature 1995; 377: 630-632.

272. Streilein JW. Unraveling immune privelege. Science 1995; 270: 1158-1159.

273. Nagata S, Golstein P. The fas death factor. Science 1995; 267: 1449-1456.

274. Alderson MR, Rough TW, Davis-Smith T, *et al.* Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med. 1995; 181: 71-77.

275. Lynch DH, Ramsdell F, Aldersob MR. Fas and Fas l in the homeostatic regulation of immune responses. Immunol Today 1995; 16: 569-574.

276. Korbutt GS, Elliott JF, Rajotte RV. Cotransplantation of allogeneic islets with testicular cell aggregates allows long-term graft survival without systemic immunosuppression. Diabetes 1997; 46: 317-322.

277. Skinner MK. Cell-cell interactions in the testis. Endocrin Rev 1991; 12: 45-77.

278. Clark AM, Griswold MD. Expression of clusterin/sulfated glycoprotein-2 under conditions of heat stress in rat sertoli cells and a mouse sertoli cell line. J Androl 1997; 18: 257-263.

279. Murphy BF, Walker ID, Kirszbaum L, D'Apice JF. SP-40-40 - a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. J Clin Invest 1988; 81: 1858-1864.

280. Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, D'Apice AJF. SP-40-40 is an inhibitor of C5b-6 initiated hemolysis. Int J Immunol 1989; 1: 551-554.

281. Wilson MR, Roeth PJ, Easterbrook-Smith SB. Clusterin enhances the formation of insoluble immune complexes. Biochem Biophys Res Commun 1991; 177: 985-990.

282. Roberts AB, Sporn MB. Transforming growth factor beta. Adv Can Res 1988; 51: 107-145.

283. Lacy PE. Treating diabetes with transplanted cells. Sci Am 1995; 273: 54-58.

284. Colton CK. Engineering challenges in cell encapsulation technology. Trends Biotechnology 1996; 14: 158-162.

285. Ao Z, Korbutt GS, Warnock GL, *et al*. Microencapsulation improves canine islet survival *in vivo*. Transplant Proc 1995; 27: 3349.

286. Soon-Shiong P, Heintz RE, Meredith N, *et al.* Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. Lancet 1994; 343: 950-951.

287. Sun Y, Ma X, Zhou D, Vacek I, Sun AM. Normalization of diabetes in spontaneously diabetic cynomolgus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. J Clin Invest 1996; 98: 1417-1422.

288. Brissova M, Petro M, Lacik I, *et al.* Evaluation of microcapsule permeability via inverse size exclusion chromatography. Anal Biochem 1996; 242: 104-111.

289. De Vos P, De Haan BJ, Wolters GHJ, *et al.* Improved biocompatibility but limited graft survival after purification of alginate for microencapsulation of pancreatic islets. Diabetologia 1997; 40: 262-270.

290. De Vos P, De Haan BJ, Van Schilfgaarde R. Upscaling the production of microencapsulated pancreatic islets. Biomaterials 1997; 18: 1085-1090.

291. Lanza RP, Chick W. Transplantation of encapsulated cells and tissues. Surgery 1997; 121: 1-9.

292. Teitelman G. On the origin of pancreatic endocrine cells, proliferation and neoplastic transformation. Tumor Biol 1993; 14: 167-173.

293. Wessels NK, Evans J. Ultrastructural studies of early morphogenesis and cytodifferentiation in the embryonic mammalian pancreas. Dev Biol 1968; 17: 413-446.

294. Pictet R, Rutter WJ. Development of the embryonic pancreas. In: Steiner DF, Frenkel N, eds. Handbook of Physiology. Washington: American Physiological Society, 1972: 25-66.

295. Hellerstrom C. The life story of the pancreatic B cell. Diabetologia 1984; 26: 393-400.

296. Swenne I. Pancreatic beta-cell growth and diabetes mellitus. Diabetologia 1992; 23: 525-528.

297. Jiang F-X, Cram DS, DeAizpurua HJ, Harrison LC. Laminin-1 promotes differentiation of fetal mouse pancreatic β-cells. Diabetes 1999; 48: 722-730.

298. Swenne I, Eriksson U. Diabetes in pregnancy: islet cell proliferation in the fetal rat pancreas. Diabetologia 1982; 23: 525-528.

299. Conklin JL. Cytogenesis of the human fetal pancreas. Am J Anat 1962; 111: 181-193.

300. Cantenys D, Portha B, Dutrillaux MC, Hollande E, Roze C, Picon L. Histogenesis of the endocrine pancreas in newborn rats after destruction by streptozotocin: an immunocytochemical study. Virchows Arch B Cell Pathol 1981; 35: 109-122.

301. Teitelman G, Joh TH, Reis DJ. Transformation of catecholaminergic precursors into glucagon (A) cells in mouse embryonic pancreas. Proc Natl Acad Sci USA 1981; 78: 5225-5229.

302. Teitelman G, Lee J, Reis DJ. Differentiation of prospective mouse pancreatic islet cells during development in vitro and during regeneration. Dev Biol 1987; 120: 425-433.

303. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982; 31: 11-24.

304. Oosterwijk E, Muijen GNP, Oosterwijk-Wakka JC, Warnaar SO. Expression of intermediate-sized filaments in developing and adult kidney and in renal cell carcinoma. J Histochem Cytochem 1990; 38: 385-392.

305. Franke WW, Jahn L, Knapp AC. Cytokeratins and desmosomal proteins in certain epithelioid and nonepithelial cells. In: Osborn M, Webber K, eds. Cytoskeletal Proteins in Tumor Diagnosis. Cold Spring Harbor, New York: Cold Spring Harbor Laboratories, 1989: 151-172.

306. Ramaekers F, Huysmans A, Schaart G, *et al.* Tissue distribution of keratin 7 as monitored by a monoclonal antibody. Exp Cell Res 1987; 170: 235-249.

307. Kasper M, Hahn von Dorsche H, Stosiek P. Changes in the distribution of intermediate filament proteins and collagen IV in fetal and adult human pancreas. I. Localization of cytokeratin polypeptides. Histochem J 1991; 96: 271-277.

308. Schussler MH, Skoudy A, Ramaekers F, Real FX. Intermediate filaments as differentiation markers of normal pancreas and pancreas cancer. Am J Pathol 1992; 140: 559-568.

309. Alpert S, Hanahan D, Teitelman G. Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 1988; 53: 295-308.

310. Herrera P-L, Huarte J, Sanvito F, *et al.* Embryogenesis of the murine endocrine pancreas: Early expression of pancreatic polypeptide gene. Development 1991; 113: 1257-1265.

311. Gittes GK, Rutter WJ. Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci USA 1992; 89: 1128-1132.

312. Teitelman G, Alpert S, Polak JM, *et al.* Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 1993; 118: 1031-1039.

313. Peshavaria M, Stein R. PDX-1: An activator of genes involved in pancreatic development and islet gene expression. In: Sarvetnick N, ed. Pancreatic growth and regeneration. Basel: Karger Landes System, 1997: 96-105.

314. Leonard J, Peers B, Johnson T, *et al.* Characterization of somatostatin transactivating factor-1, a novel homeobox factor that stimulates somatostatin expression in pancreatic islet cells. Mol Endocrinol 1993; 7: 1275-1283.

315. Ohlsson H, Karlsson K, Edlund T. IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J 1993; 12: 4251-4259.

316. Miller CP, McGehee R, Habener JF. IDX-1: A new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. EMBO J 1994; 13: 1145-1156.

317. MacFarlane W, Read ML, Gilligan M, Bujalska I, Docherty K. Glucose modulates the binding activity of the β cell transcription factor IUF-1 in a phosphorylation-dependent manner. Biochem J 1994; 303: 625-631.

318. Stoffers DA, Thomas MK, Habener JF. Homeodomain protein IDX-1: A master regulator of pancreas development and insulin gene expression. TEM 1997; 8: 145-151.

319. Zangen DH, Miller CP, Smith FE, *et al.* Increased islet and ductal insulin promoter factor/IDX-1 expression in pancreatic regeneration. Proc Eur Assoc Study Diabet Abst 1995; 46: 258-264.

320. Finegood DT, Scaglia L, Bonner-Weir S. Dynamics of β-cell mass in the growing rat pancreas: estimation with a simple mathematical model. Diabetes 1995; 44: 249-256.

321. Ahlgren U, Pfaff SL, Jessel TM, *et al.* Independent requirement for ISL-1 in formation of pancreatic mesenchyme and islet cells. Nature 1997; 385: 257-260.

322. Sosa-Pineda B, Chowdhury K, Torres M, *et al.* The pax4 gene is essential for differentiation of insulin-producing β cells in the mammalian pancreas. Nature 1997; 386: 399-402.

323. St-Onge L, Sosa-Pineda B, Chowdhury KM, *et al.* Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature 1997; 387: 406-409.

324. Naya FJ, Huang H-P, Qiu Y, *et al.* Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/NeuroD-deficient mice. Genes Dev 1997; 11: 2323-2334.

325. Satake M, Kumagai-Braesch M, Korsgren O, *et al*. Characterization of humoral human anti-porcine xenoreactivity. Clin Transplant 1993; 7: 281-288.

326. Hamelmann W, Gray DWR, Cairns TDJ, *et al*. Immediate destruction of xenogeneic islets in a primate model. Transplantation 1994; 58: 1109-1114.

CHAPTER II

EXPRESSION OF GALα(1,3)GAL ON NEONATAL PORCINE ISLET BETA CELLS AND SUSCEPTIBILITY TO HUMAN ANTIBODY-COMPLEMENT LYSIS[1]

II-A) INTRODUCTION

Endocrine replacement by islet transplantation is an attractive alternative treatment for patients with type 1 diabetes. Transplantation of pancreatic islets in patients with diabetes has been shown to induce a state of normoglycemia (1-3) and long-term insulin independence can be achieved when sufficient number of islets were transplanted. Widespread clinical application of this treatment however, has been hampered by the shortage of cadaveric organs for transplantation. One possible solution to this problem is to use islet tissue from an abundant and accessible animal source. Pigs are very appealing source of islets because they breed rapidly and share many biological features with humans including the similarity in insulin structure (4). Despite many reports on the isolation of adult porcine islets, several factors such as age, breed, and quality of organs adversely affect the final yield (5,6) with the additional problem that once isolated, adult porcine islets are fragile and difficult to maintain in tissue culture (7-9). A more attractive source of tissue that does not have the same problem associated with adult pig is the neonatal pig. Recently, Korbutt *et al.* (10) developed a method to isolate large numbers of culture, NPI were shown to correct diabetes in nude mice and exhibit growth both *in vitro* and *in vivo* (10).

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[1] *A version of this chapter has been published. Rayat et al. 1998. Diabetes 47: 1406.*

Although NPI constitute an attractive source of insulin-producing tissue for transplantation, the issue of humoral-mediated xenograft rejection (*i.e.* hyperacute rejection) of such implants needs to be addressed. This rejection process is initiated when naturally occurring xenoreactive antibodies in recipient sera bind to antigens present on the surface of endothelial cells of donor organs. Antibody binding in turn activates complement, which rapidly destroys the transplanted organ or tissue. The most important target for these antibodies has been identified as the carbohydrate Galα(1,3)Galβ(1,4)GlcNAc-R or Galα(1,3)Gal epitope (11-13). This epitope is present in high concentrations on all porcine endothelial cells (13-15) and although it has been detected on fetal porcine islet cell clusters (16,17) the expression of Galα(1,3)Gal on NPI has not been elucidated. Moreover, current literature suggests that Galα(1,3)Gal is not expressed on fully differentiated islet endocrine cells, but rather on intra-islet ductal and endothelial cells (16-19). However, it has been demonstrated that natural xenoreactive antibodies bind to fetal (18), neonatal (20), and adult (21,22) porcine islet cells, and exposure to human sera containing active complement *in vitro* results in rapid destruction of these tissues (20-22). In this study we examined the expression of Galα(1,3)Gal on cells present in NPI and related this expression to the susceptibility of these cells to human antibody/complement-mediated lysis *in vitro*.

II-B) MATERIALS AND METHODS

1. Preparation of Neonatal Porcine Islets

The method used to prepare NPI has been previously described (10). Briefly, 1 to 3 day-old Landrace-Yorkshire neonatal pigs (1.5-2.0 kg body weight) of either sex were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, cut into small pieces, and digested with 2.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO). After filtration through a nylon screen (500 μm), the tissue was cultured for 9 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (BSA; fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 μg/ml streptomycin.

2. Immunohistochemical Analysis

The expression of Galα(1,3)Gal epitope and presence of insulin-positive cells within intact neonatal porcine pancreas and NPI was assessed by double immunohistochemistry staining. All samples were fixed in Bouin's solution for 2 hours, washed three times with 70% ethanol, and embedded in paraffin. Sections (5μm) were first stained with biotinylated BS-1 isolectin B4 from *Bandeiraea simplicifolia* BS-1 (1:25 dilution; Sigma) (14, 15) for 30 minutes at room temperature to detect the presence of Galα(1,3)Gal epitope. The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories, Burlingame, CA) method was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) to produce a brown color. The same tissue sections were subsequently stained with guinea pig anti-porcine insulin antibody (1:1,000 dilution; Dako Laboratories Canada Inc., Mississauga, ON) for 30 minutes followed by biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories) for 20 minutes. The avidin-biotin complex /alkaline phosphatase method (ABC/AP; Vector Laboratories) and Fast Red as chromagen (Vector Laboratories) was used to obtain a red reaction. Paraffin-embedded sections of porcine aorta and porcine aortic endothelial cells (PAEC; Cell Systems, Kirkland, WA) placed on poly-L-lysine coated slides were used as positive controls since they are both known to express Galα(1,3)Gal. As expected, the vascular endothelium and isolated PAEC stained positive for Galα(1,3)Gal antigen. Negative controls for the staining techniques consisted of omission of the primary antibodies as well as sections of paraffin-embedded human pancreas. These tests resulted in negative staining reactions.

3. Characterization of Dissociated NPI Cells

To further define the cellular specificity of Galα(1,3)Gal expression, 9-day culture NPI were dissociated into single cells by gentle agitation in calcium-free media containing 15 μg/ml trypsin (Boehringer Mannheim, Laval, PQ) and 4 μg/ml DNAse (Boehringer Mannheim). The cell suspension was filtered through a 63 μm nylon screen to remove cell clumps and then centrifuged through Percoll (Sigma) of density 1.040 g/ml to eliminate dead cells and debris. Single cell ($10\text{-}15 \times 10^6$) suspensions were subsequently incubated with fluorescein isothiocyanate (FITC)-conjugated BS-1 isolectin B4 (1:50; Sigma) for 1 hour on ice, washed, and submitted to FACS (EPICS Elite ESP flow cytometer, Coulter, Hialeah, FL) analysis for the purification of Galα(1,3)Gal-positive and -negative islet cells. The fluorescence emitted by FITC-labeled cells was selected as the sorting parameter. Positive controls for these experiments, included PAEC stained with FITC-conjugated IB4 lectin using the same protocols as for NPI. The efficacy of the cell separation was evaluated by comparing visually the fluorescence of sorted Galα(1,3)Gal-positive and -negative cells, as well as by re-analysing each population by flow cytometry to detect relative percentage of Galα(1,3)Gal-positive cells in each population. The cellular composition of freshly dissociated and sorted cell populations was determined by immunohistochemical staining for insulin (as described above) and glucagon following adherence of the cells on poly-L-lysine coated slides and fixation in Bouin's. Glucagon-positive cells were detected using rabbit anti-porcine glucagon antibody (1:1,000; Dako Diagnostics Canada Inc.) followed by biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories) and DAB as chromagen (BioGenex).

For detecting the binding of preformed human natural xenoreactive antibodies to NPI, $1x10^6$ cells were incubated in varying dilutions (1:2, 1:8, 1:16, and 1:32) of heat-inactivated (HI) pooled human AB serum (NABI, Miami, FL) for 1 hour on ice. The cells were then washed, labeled with FITC-conjugated rabbit anti-human IgG or IgM (1:10; Dako Diagnostics Canada Inc.) for 1 hour on ice, washed and the percentage of cells binding to human antibodies was determined by FACS analysis.

4. Cytotoxicity Assay and Morphology

To determine the susceptibility of NPI cells to antibody/complement-mediated lysis, fractions of unsorted and FACS-purified Galα(1,3)Gal-positive and -negative cells were exposed to fresh human AB serum collected from one donor or HI-pooled human AB serum (NABI) with the addition of rabbit complement (Pel-Freeze, Brown Deer, WI) obtained from 3 to 4 week-old rabbits. This complement was selected since rabbits at this age are believed not to exhibit xenoreactive antibodies to other species such as the pig. To confirm the absence of xenoreactive antibodies in this complement, we incubated dissociated NPI in HI-rabbit complement (1:5 dilution; Pel-Freeze) for 1 hour on ice. Positive controls for this experiment included NPI incubated in HI-normal adult rabbit serum using the same protocol. Islet cells were then washed and stained with either R-phycoerythrin-conjugated goat anti-rabbit IgG (1:100 dilution; Vector Laboratories) or mouse anti-rabbit IgM (0.44 mg/ml; Serotec Canada, Mississauga, ON) for 30 minutes on ice. Rabbit IgM antibody binding on NPI cells was detected by further incubating the cells with Cy™ 3-conjugated goat anti-mouse antibody (1:100 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 minutes on ice. Stained cells were washed and analysed by flow cytometry. Positive controls (i.e. adult rabbit serum) demonstrated that >90% of the islet cells bound both rabbit IgG and IgM (Appendix). In contrast, NPI incubated in rabbit complement exhibited <1% binding of rabbit IgG or IgM (Appendix). These results thus confirm that complement obtained from 3 to 4 week-old rabbits contains no xenoreactive antibodies to NPI.

Prior to the cytotoxicity assay, single islet cell suspensions were distributed over poly-L-lysine-coated 24-well plates ($5x10^4$ cells/well) and cultured overnight in 1 ml of HAM's F10 media (supplemented as previously described). Five hundred microliters of media was then removed from each well, replaced with the same amount of human serum (final concentration 50%, vol/vol), incubated for 1 hour at 37 °C, then 200 µl of media was removed and replaced with rabbit complement and further incubated for 30 minutes at 37 °C. When fresh human serum was used (i.e. containing active complement), no rabbit complement was added to the well. Controls included cells incubated in media or HI serum only. After incubation, 60 µl of 2.5 mg/ml (final concentration 0.01%, wt/vol) stock neutral red solution (Fisher Scientific Co., Edmonton, AB) was added to each well for 30 minutes and the number of neutral red-positive (live) and negative cells (dead) was determined. The percentage of total dead cells was calculated as follows:

$$\%\ \text{dead cells} = \frac{\text{cell viability (controls)} - \text{cell viability (serum +complement)}}{\text{cell viability (controls)}} \times 100$$

The ultrastructure of NPI after exposure to human serum and complement was examined by electron microscopy as previously described (10). Briefly, cells were fixed in 2.5% (vo/vol) glutaraldehyde (Millonig's buffer, pH 7.2), pot-fixed in 1.5% (wt/vol) OsO4, washed in distilled water, then dehydrated successively in 50, 70, 80, 90, 100% ethanol, before embedding in araldite. Sections were stained with lead citrate and uranyl acetate then subsequently examined in Hitachi H 7000 (Hitachi Ltd., Tokyo, Japan) transmission electron microscope.

5. Statistical Analysis

Data are expressed as means±SEM of n independent experiments. Statistical significance of differences among various groups was determined using one-way analysis of variance (ANOVA) and between two groups by paired T-test analysis. $p<0.05$ was considered significant.

II-C) RESULTS

1. Expression of Galα(1,3)Gal on Neonatal Porcine Pancreas and Islet Cells

The expression of Galα(1,3)Gal epitope within intact neonatal porcine pancreas was localized on the lumen of vascular endothelium and pancreatic ducts (Figure 2-1A). Insulin-positive β cells were scattered randomly in the pancreas as either single cells or small clusters frequently arranged alongside or within the duct lining. The cell surface of a small proportion of these β cells were stained positive for Galα(1,3)Gal epitope. In freshly digested pancreatic fragments Galα(1,3)Gal was detected on capillary/ductal fragments and on the majority of insulin-positive β cells (Figure 2-1B). Following 9 days culture the cellular aggregates developed into spherical structures similar to adult pancreatic islets. Immunohistochemical analysis of these aggregates showed an increased staining for Galα(1,3)Gal localized along the islets' periphery as well as in the lumen of intra-islet ductal elements (Figure 2-1C). Although the percentage of insulin-positive cells increased during culture, the proportion of β cells expressing Galα(1,3)Gal appeared to decrease. To more accurately demonstrate whether a proportion of pancreatic β cells express Galα(1,3)Gal, single cell suspensions of 9-day culture islets were also examined. Immunohistochemical analysis of these preparations clearly demonstrated Galα(1,3)Gal expression on a proportion of the insulin-positive cells (Figure 2-1D).

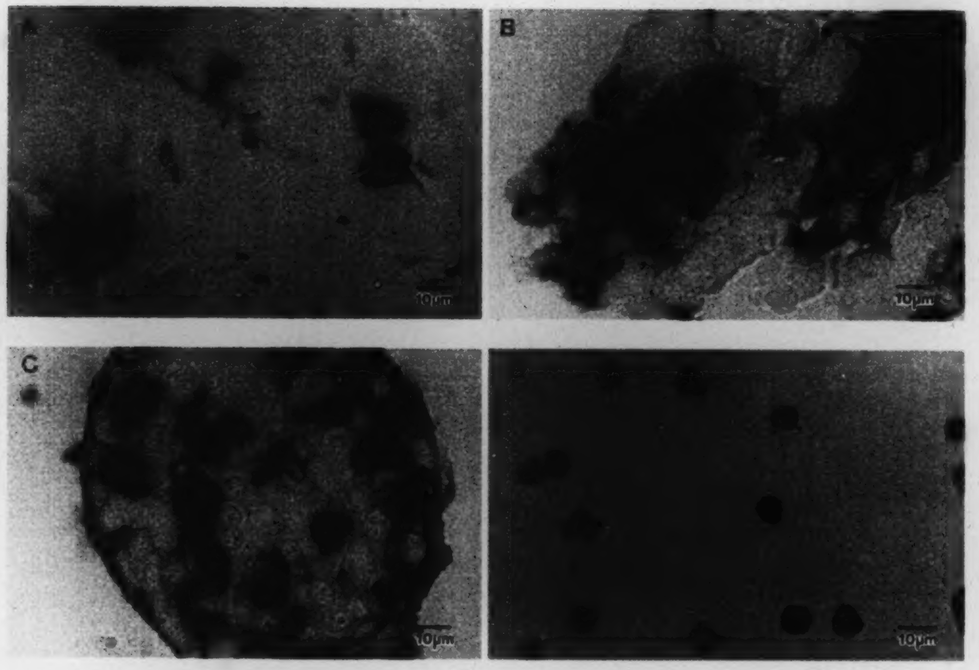

Figure 2-1. Light micrographs of native neonatal porcine pancreas (A), freshly digested neonatal porcine pancreatic aggregates (B), 9-day culture islets (C), and 9-day-culture dissociated islet cells (D). Sections were double immunohistochemically stained for Galα(1,3)Gal epitope (brown) and insulin (red). Arrows point to some double positive cells.

2. Characterization of Neonatal Porcine Islet Cells

FACS analysis of the neonatal porcine single islet cell suspensions stained with FITC-conjugated IB4 lectin demonstrated that 30.0±3.0% (n=15) of the cells expressed Galα(1,3)Gal (purity=90.0±2.1%) while 70.0±2.0% (n=15; purity=98.3±0.4%) did not (Figure 2-2 and Figure 2-3). A heterogeneity existed amongst the various pigs tested since the mean percentage of islet cells staining positive for Galα(1,3)Gal in 4 of 15 piglets was lower (range = 8.7-17.1%) than those prepared from the remaining animals (range = 20.2-48.8%). Immunohistochemical analysis of unsorted NPI cell preparations indicated the presence of 33.5±2.7% (n=12) insulin-positive and 39.1±5.0% (n=12) glucagon-positive cells (Table 2-1). The Galα(1,3)Gal-positive fractions contained, 23.1±5.1% (n=10) insulin-positive cells while 31.8±3.7% (n=10) were glucagon-positive. Similarly, the Galα(1,3)Gal-negative population was composed of 37.6±4.4% (n=12) and 31.2±4.0% (n=9) insulin and glucagon-containing cells, respectively.

The binding of preformed xenoreactive antibodies in normal human serum directed against NPI was demonstrated by FACS analysis. At all tested dilutions of human serum (1:2 to 1:32), FACS analysis revealed >90% (range=91-99%; n=4) of NPI cells bound both human IgG and IgM antibodies (Figure 2-4). When cells were incubated in the presence of FITC-labeled secondary antibodies no significant fluorescence was detected.

Table 2-1. Cellular composition of neonatal porcine islet cells.

Condition	Percentage of total cells Insulin-positive	 Glucagon-positive
Unsorted	33.5±2.7(12)	39.1±5.0(12)
Sorted		
Gal-positive	23.1±5.1(10)	31.8±3.7(10)
Gal-negative	37.6±4.4(12)	31.2±4.0(9)

Values are means±SEM of (n) independent experiments. Cell composition was determined by immunohistochemical analysis as described in Methods.

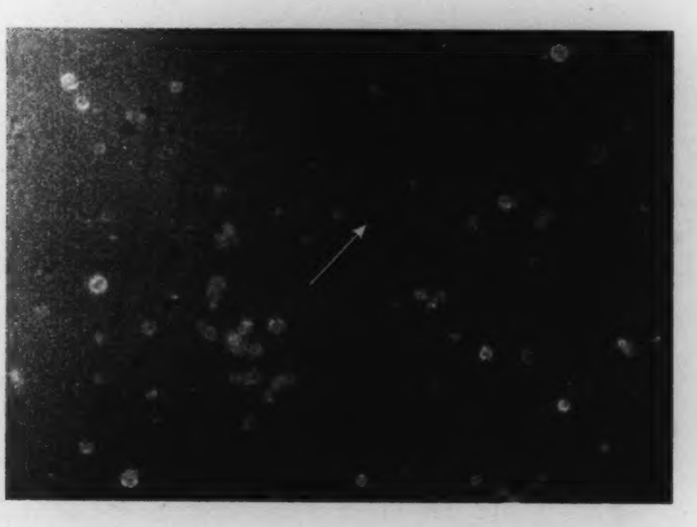

Figure 2-2. Light micrograph of NPI cells stained with FITC-conjugated IB4 lectin. Magenta arrow points to a cell which express Galα(1,3)Gal and white arrow points to a cell lacking the antigen.

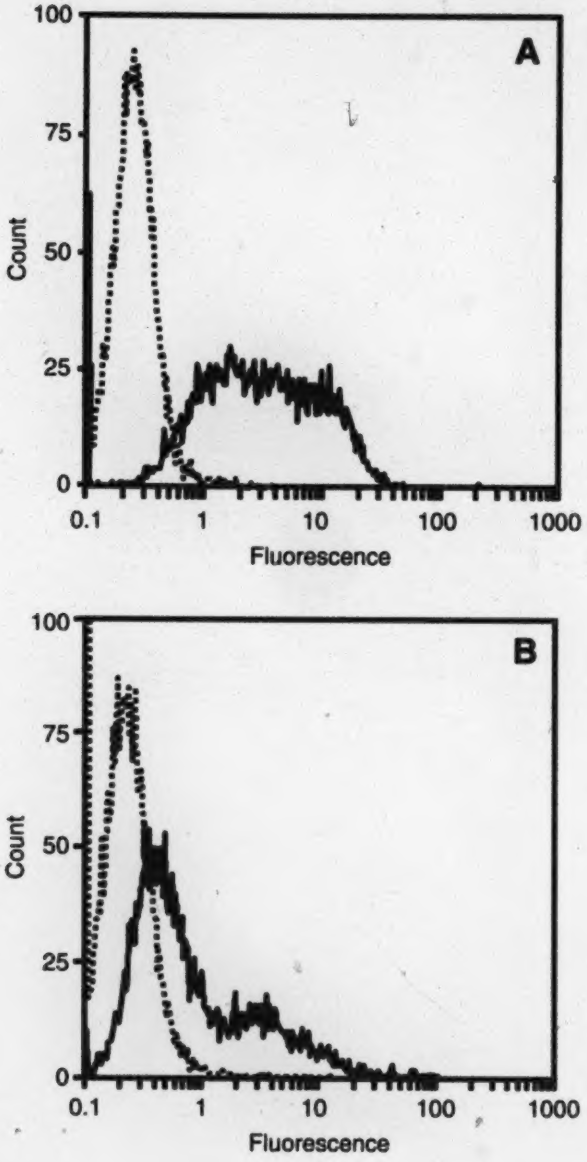

Figure 2-3. FACS analysis of PAEC (A) and dissociated 9-day culture NPI (B) stained with FITC-conjugated IB4 lectin. Dotted line represents negative controls not stained with IB4.

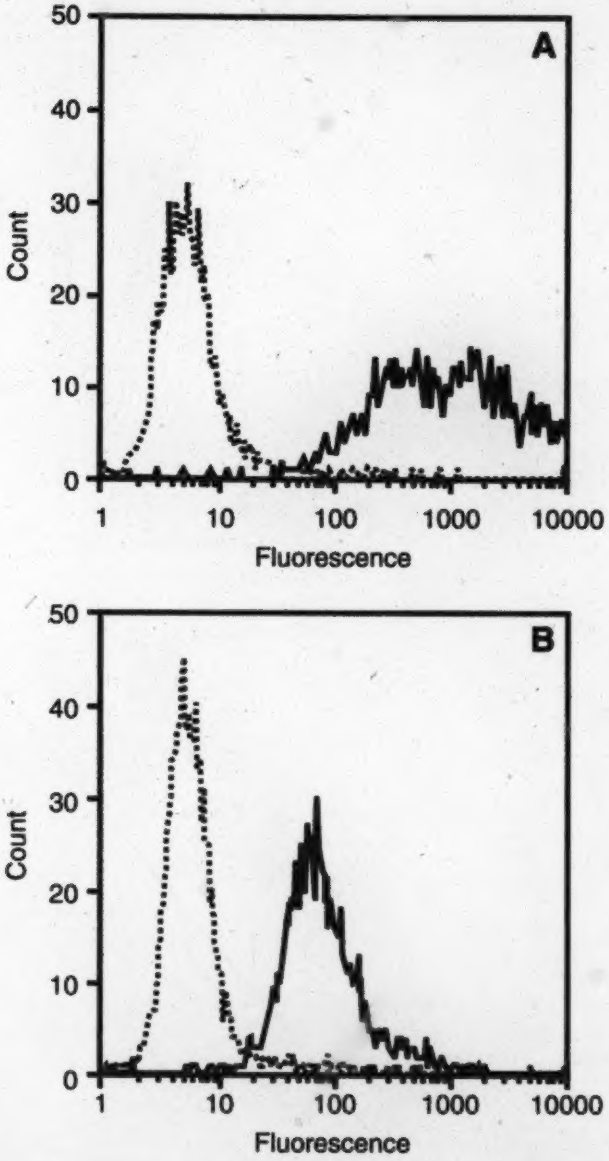

Figure 2-4. Binding of human xenoreactive antibodies to NPI. Single cell suspensions of NPI were incubated with 1:32 dilution of HI-pooled human AB serum for 1 hour then with 1:10 dilution of FITC-conjugated rabbit anti-human IgG (A) or IgM (B) for an additional 1 hour. Dotted lines represent negative control islet cells incubated in FITC-labeled secondary antibodies alone. Results are representative of 3 independent experiments.

3. Susceptibility to Antibody/Complement-Mediated Lysis

Incubation of NPI cells in the presence of heat-inactivated pooled human AB did not result to significant lysis of these cells, however when rabbit complement was added >90% of cells in unsorted as well as Galα(1,3)Gal-positive and -negative cell populations were killed (Table 2-2). Similar results were obtained in all cell fractions when exposed to rabbit complement alone. In contrast, cell preparations that had been exposed to unmodified fresh human serum contained significantly ($p<0.05$) fewer damaged cells (41-46% dead cells; Table 2-2). When a CH_{50} assay was performed to determine complement activity, the condition in which rabbit complement was added exhibited values 1.5-fold higher (CH_{50}=300 units/ml) than that measured in fresh human serum (CH_{50} = 193±12.2 units/ml; n=10). When examined by electron microscopy, incubation of islet cells in HAM's F10 media or HI serum containing no complement activity did not affect cell viability. These preparations demonstrated numerous well-granulated and ultrastructurally intact cells (Figure 2-5). In contrast, the majority of cells exposed to HI-AB serum with rabbit complement or rabbit complement only exhibited ruptured plasma membranes with condensed chromatin.

Table 2-2. Susceptibility of neonatal porcine islet cells to human antibody/complement-mediated lysis.

Condition	Percentage of dead cells Unsorted	Gal-positive	Gal-negative
HI-pooled AB serum	2.4±1.5 (13)	2.9±1.9 (8)	4.3±1.9 (13)
HI-pooled AB serum+complement	97.4±1.7(9)[a]	97.9±1.3(11)[a]	97.9±1.1(15)[a]
Complement	92.5±3.1(9)[a]	91.4±3.0(10)[a]	90.9±2.9(14)[a]
Fresh AB serum	40.7±4.9(6)[b]	46.9±7.7(4)[b]	45.7±3.2(6)[b]

Values are means±SEM of (n) independent experiments. Cells were exposed in 50% human serum for 1 hour and 20% rabbit complement for another half an hour. Fresh serum was from one individual with blood type AB. The percentage of dead cells was calculated as described in Methods. Statistical significance of differences was calculated by one-way ANOVA. a $p<0.05$ *vs* HI-pooled human AB serum; b $p<0.05$ *vs* complement or HI-pooled human AB serum + complement.

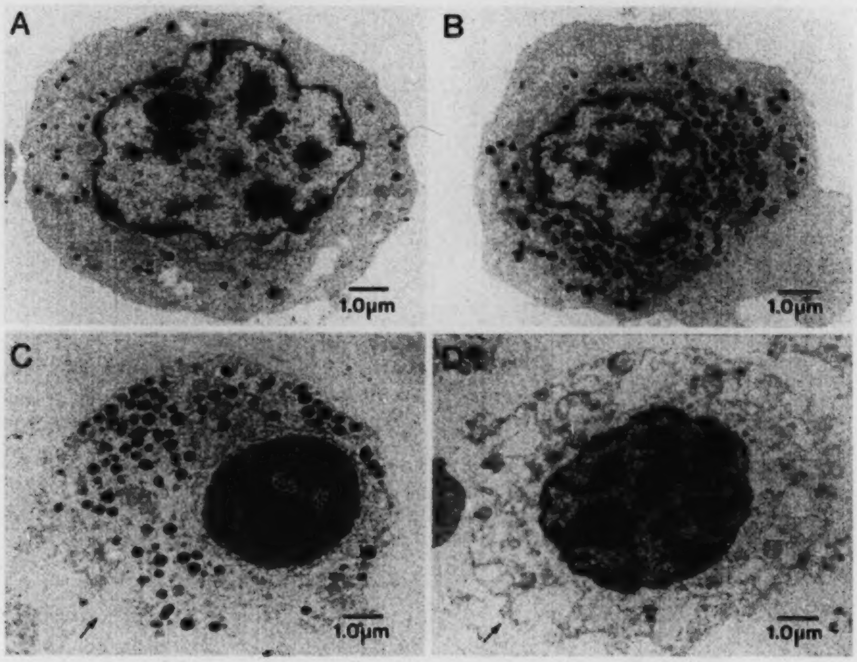

Figure 2-5. Electron micrographs of dissociated NPI cells exposed to media (A), HI-pooled human AB serum containing no complement activity (B), HI-pooled human AB serum with rabbit complement (C) or with complement only (D). Control islets (A, B) appear ultrastructurally intact and well granulated, whereas those exposed to complement (+/- serum) exhibited ruptured plasma membranes (arrows) with condensed chromatin.

II-D) DISCUSSION

NPI constitute an attractive source of xenogeneic insulin-producing tissue for clinical transplantation, however, several aspects of the model need further investigation before this tissue can be considered for treating patients with type 1 diabetes. In particular, the susceptibility of porcine pancreatic endocrine cells to immunologic destruction *via* complement activation has not been fully elucidated. In the present study, neonatal porcine pancreatic islet cells were characterized for expression of the xenoreactive Galα(1,3)Gal epitope and sensitivity to human antibody/complement-mediated lysis *in vitro*. The data presented here demonstrate that Galα(1,3)Gal residues are expressed in neonatal porcine pancreata and that this epitope is detected following collagenase digestion and 9-day culture of islet cell aggregates. In the native pancreas, Galα(1,3)Gal was detected on vascular endothelium, within ductal lumen, and on the surface of insulin-positive β cells. Immunohistochemical staining of freshly digested pancreatic fragments, 9-day cultured NPI, and single islet cell suspensions revealed that a proportion of insulin containing β cells clearly expressed Galα(1,3)Gal. To further identify which pancreatic islet cells express Galα(1,3)Gal, we used the lectin IB4 to FACS purify cells with detectable levels of Galα(1,3)Gal. It was shown that approximately 30% of neonatal porcine islet cells express Galα(1,3)Gal residues and that 55% of these cells stained positive for either insulin or glucagon. Thus, approximately 17% of the total porcine NPI cell population can be characterized as Galα(1,3)Gal expressing insulin/glucagon-positive cells. On the other hand, majority of NPI cells (70%) were shown not to express Galα(1,3)Gal, and that 69% of this population is composed of insulin and glucagon-positive cells. Heterogeneity therefore exists amongst NPI cells, in that a sub-population of non-endocrine as well as endocrine cells express Galα(1,3)Gal.

This study also demonstrates that Galα(1,3)Gal is highly expressed in developing NPI cells, and that the proportion of insulin-producing β cells staining positive for Galα(1,3)Gal residues appear greater in freshly isolated preparations as compared to those allowed to mature further during the 9-day culture period. Furthermore, morphological assessment of NPI grafts at >200 days post-transplantation in diabetic nude mice (*i.e.* following further maturation), revealed implants predominantly composed of insulin-positive cells with Galα(1,3)Gal reactivity restricted to mouse endothelial cells originating from revascularization of the grafts (data shown in Chapter III). It is therefore possible that Galα(1,3)Gal is expressed on a subpopulation of less mature NPI cells that are either precursors of hormone secreting cells or transitional cytodifferentiated forms of insulin/glucagon containing cells that eventually lose Galα(1,3)Gal expression once becoming fully differentiated and/or functionally mature.

The results presented here differ from those obtained in other studies, suggesting that Galα(1,3)Gal is not present on fetal (17) or adult (17,19) pancreatic endocrine cells but limited to intra-islet ductal and endothelial cells (15,17,19). Since these data indicate that Galα(1,3)Gal is expressed on immature/developing β cells, this possibly explains why Mirenda *et al.* (19) did not detect Galα(1,3)Gal on adult porcine β cells but it remains uncertain why McKenzie *et al.* (17) did not observe Galα(1,3)Gal residues on fetal porcine endocrine cells. It is possible that differences in experimental design as well as age, strain and even from one individual animal to another may account for this variability in Galα(1,3)Gal expression. For example, the islet cells isolated from Yorkshire-Landrace pigs (used in this study) exhibited variability in Galα(1,3)Gal expression. Similarly, Geller *et al.* (23) reported variation in porcine xenogeneic antigen expression amongst pigs within the same as well as different strains.

Using an *in vitro* cytotoxicity assay, approximately 50% of NPI cells are rapidly lysed by the activation of human complement. Exposure to HI human serum containing rabbit complement led to the lysis of more than 90% of the islet cells. Since rabbit complement was tested not to contain xenoreactive antibodies, the increased cytotoxicity seen in this condition is likely due to higher levels of complement activity than that measured in fresh human serum. Furthermore, the present study confirms Korbutt *et al.* previous data using intact NPI (20) and are in agreement with those previously reported with fetal (18) and adult (21,22) porcine islet cells. In contrast, Mirenda *et al.* (19) has recently shown no cytotoxic effect or alteration in adult porcine islet function despite binding of human IgG or IgM antibodies and deposition of complement. It is possible that differences in age and strain of the pigs as well as selection of cytotoxicity protocols, may account for these differences. For example, previous reports that examined porcine islet viability after exposure to human sera provide no data regarding complement activity in the sera tested (18,19,22). It is therefore possible that an observed lack of islet cell toxicity is simply related to diminished complement activity during collection and/or the time required to pool sera from multiple donors. In order to exclude the possible loss of complement activity during storage freshly prepared serum from one individual or HI serum (i.e. as a source of xenoreactive antibodies) supplemented with rabbit complement was used.

The observation that NPI cells were destroyed in the absence of xenoreactive antibodies with rabbit complement alone are in agreement with other *in vitro* studies using either porcine endothelial (24) or adult islet (22) cells. Although, the role of classical and alternative pathways in complement activation within different species combinations remains controversial (24-29), several other xenotransplant models have also demonstrated hyperacute rejection of discordant xenografts mediated by complement activation *via* the alternative pathway (28,29). In these cases, HAR can occur in the absence of xenoreactive antibodies and is very aggressive, perhaps because the kinetics of complement activation is independent of antibody binding. The present study suggests that Galα(1,3)Gal expression on NPI cells might not be a critical factor in inducing complement-mediated lysis since majority (>90%) of NPI cells were killed after treatment with complement alone. Moreover, NPI cells lacking Galα(1,3)Gal are also killed. It is possible that Galα(1,3)Gal-negative cells may be lysed by two different mechanisms namely, by binding of xenoreactive antibodies to non-Galα(1,3)Gal epitopes (causing complement activation) or by activation of the alternative complement pathway. Several reports have suggested that human preformed anti-Galα(1,3)Gal antibodies are the major, if not exclusive, xenoreactive antibodies responsible for hyperacute rejection (11,30-32) and that adsorption of these antibodies may reduce the serum's cytotoxicity (33,34). However, it has also been shown that human anti-Galα(1,3)Gal antibodies are capable of recognizing alternative ligands which still remain uncharacterized (35). In addition, Oriol *et al.* (14) reported that carbohydrate epitopes with terminal lactosamine (βGal1-4GlcNAc) or sialic acid (αNeuAc2-3βGal1-4GlcNAc) are also present on porcine vascular endothelium, suggesting the possibility that other xenoreactive antibodies are directed against different carbohydrate epitopes.

In summary, this study shows that NPI are composed of cells expressing Galα(1,3)Gal, and that expression of this epitope is not restricted to non-endocrine cells. Furthermore, cells that express or lack Galα(1,3)Gal are both susceptible to complement-mediated lysis. This form of immunologic destruction takes place even in the absence of preformed natural antibodies, indicating a key role of the alternative pathway of complement system in the destruction of NPI cells *in vitro*. Taken together, these results suggest that NPI grafts may be subjected to complement-mediated destruction if implanted into patients with type 1 diabetes, however, this must be confirmed in an *in vivo* model.

II-E) REFERENCES

1. Ricordi C, Tzakis AG, Carroll PB, *et al.* Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992; 53: 407-414.

2. Scharp DW, Lacy PE, Santiago JV, *et al.* Results of our first nine intraportal islet allografts in type 1, insulin-dependent diabetic patients. Transplantation 1991; 51: 76-85.

3. Warnock GL, Kneteman NM, Ryan EA, *et al.* Continued function of pancreatic islets after transplantation in type 1 diabetes. Lancet 1989; 11: 570-572.

4. Cooper DKC, Ye Y, Rolf LL Jr, Zuhdi N. The pig as a potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. Xenotransplantation: The transplantation of organs and tissues between species, 1st ed. New York: Springer-Verlag, 1991: 481-499.

5. Socci C, Ricordi C, Davalli AM, *et al.* Selection of donors significantly improves pig islet isolation yield. Horm Metab Res 1989; 25 (Suppl. 1): 32-35.

6. Kirchhof N, Hering BJ, Geiss V, *et al.* Evidence for breed-dependent differences in porcine islets of langerhans. Transplant Proc 1994; 26:616-617.

7. Ricordi C, Socci C, Davalli AM, *et al.* Isolation of the elusive pig islet. Surgery 1989; 107: 688-694.

8. Van Deijnen JHM, Hulstaert CE, Wolters GHJ, Van Shilfgaarde R. Significance of the peri-insulinar extracellular matrix for islet isolation from the pancreas of the rat, dog, pig and man. Cell Tissue Res 1992; 267: 139-146.

9. Marchetti P, Finke EH, Swanson C, *et al.* The potential of porcine islet xenotransplantation in the therapy of diabetes. Diab Nutr Metab 1992; 5 (Suppl. 1): 151-154.

10. Korbutt GS, Elliott JF, Ao Z, *et al.* Large scale isolation, growth and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-2129.

11. Galili U, Rachmilewitz EA, Peleg A, Flechner I. A unique natural human IgG antibody with anti-α-galactosyl specificity. J Exp Med 1984; 160: 1519-1531.

12. Galili U. Interaction of natural anti-Gal antibody with α galactosyl epitopes: A major obstacle for xenotransplantation in humans. Immunol Today 1993; 14:480-482.

13. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC. Anti-pig IgM antibodies in human serum react predominantly with Galα(1-3)Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391-11395.

14. Oriol R, Ye Y, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433-1442.

15. McKenzie IFC, Xing PX, Vaughan HA, *et al.* Distribution of the major xenoantigen (gal(α1-3)gal)for pig to human xenografts. Transplant Immunol 1994; 2: 81-86.

16. Rydberg L, Groth CG, Moller E, *et al.* Is the Galα(1,3)Gal epitope a major target for xenoantibodies on pig fetal islet cells? Xenotransplantation 1995; 2: 148-153.

17. McKenzie IFC, Koulmanda M, Mandel TE, *et al.* Pig-to-human xenotransplantation: The expression of Galα(1,3)Gal epitopes on pig islet cells. Xenotransplantation 1995; 2: 1-7.

18. Satake M, Kumagai-Braesch M, Korsgren O, *et al.* Characterization of humoral human anti-porcine xenoreactivity. Clin Transplantation 1993; 7: 281-288.

19. Mirenda V, Le Mauff B, Cassard A, *et al.* Intact pig pancreatic islet function in the presence of human xenoreactive natural antibody binding and complement activation. Transplantation 1997; 63: 1452-1462.

20. Korbutt GS, Aspeslet LJ, Rajotte RV, *et al.* Natural human antibody-mediated destruction of porcine neonatal islet cell grafts. Xenotransplantation 1996; 3: 207-216.

21. Schaapherder AFM, Wolvekamp MCJ, Te Bulte MTJW, *et al.* Porcine islets of Langerhans are destroyed by human complement and not by antibody-dependent cell-mediated mechanisms. Transplantation 1996; 62: 29-33.

22. Schaapherder AFM, Daha MR, Van Der Woude FJ, *et al.* IgM, IgG, and IgA antibodies in human sera directed against porcine islets of Langerhans. Transplantation 1993; 56: 1576-1578.

23. Geller RL, Rubenstein P, Platt JL. Variation in expression of porcine xenogeneic antigens. Transplantation 1994; 58: 272-277.

24. Zhao Z, Termignon JL, Cardoso J, *et al.* Hyperacute xenograft rejection in the swine-to-human donor-recipient combination: *In vitro* analysis of complement activation. Transplantation 1994; 57: 245-249.

25. Auchincloss H Jr. Xenogeneic transplantation: A review. Transplantation 1988; 46: 1-20.

26. Gambiez L, Salame E, Chereau C, *et al.* The role of natural IgM in the hyperacute rejection of discordant heart xenografts. Transplantation 1992; 54: 577-583.

27. Platt JL, Fischel RJ, Matas AJ, *et al.* Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 214-220.

28. Miyagawa S, Hirose H, Skirakura Y, *et al.* The mechanism of discordant xenograft rejection. Transplantation 1995; 46: 825-830.

29. Johnston PS, Wang MW, Lim SML, *et al.* Discordant xenograft rejection in an antibody-free model. Transplantation 1992; 54: 573-576.

30. Vaughan HA, Loveland BE, Sandrin MS. Galα(1,3)Gal is the major xenoepitope expressed on pig endothelial cells recognized by naturally occurring cytotoxic human antibodies. Transplantation 1994; 58: 879-882.

31. Lawson JH, Platt JL. Molecular barriers to xenotransplantation. Transplantation 1996; 62: 303-310.

32. Good AH, Cooper DKC, Malcolm AJ, *et al.* Identification of carbohydrate structures that bind human antiporcine antibodies: Implications for discordant xenografting in humans. Transplant Proc 1992; 24: 559-562.

33. Ye Y, Neethling FA, Niekrasz M, *et al.* Evidence that intravenously administered a-galactosyl carbohydrates reduce baboon serum cytotoxicity to pig kidney cells (PK15) and transplanted pig hearts. Transplantation 1994; 58: 330-337.

34. Neethling FA, Koren E, Ye Y, *et al.* Protection of pig kidney (PK15) cells from the cytotoxic effect of anti-pig antibodies by α-galactosyl oligosaccharides. Transplantation 1994; 57: 959-963.

35. Sanchez JA, Michler RE, Rose EA, Cooper DKC. Experimental xenotransplantation between closely related primate species. In: Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. Xenotransplantation: The transplantation of organs and tissues between species, 1st ed. New York: Springer-Verlag, 1991: 365-375.

CHAPTER III

GALα(1,3)GAL EXPRESSION ON PORCINE ISLET CELLS IS AGE-DEPENDENT

III-A) INTRODUCTION

The observation in 1967 that isolated rat islets when transplanted into syngeneic animals were capable of reversing experimentally induced diabetes and its associated side effects was considered a revolutionary breakthrough in the field of islet transplantation (1). Prolonged islet cell survival across allogeneic barriers along with improved methods of isolation and the availability of more effective immunosuppressive agents prompted the initiation of clinical trials which mandated the use of isolated islets to reverse hyperglycemia in patients with type 1 diabetes (1). As a result, both short-term and long-term insulin independence following human islet allotransplantation has been reported and has encouraged many centres in the world to continue clinical trials of this form of therapy for diabetic patients (2-8). However, the critical shortage of human donor tissue is one of the major factors that limits the wide clinical application of this procedure. A potential solution to this problem is the use of non-human donor islets such as pigs. Porcine islets represent the most likely practical alternative source of insulin-producing tissue since pigs are inexpensive, readily available, and exhibit physiological and morphological characteristics comparable to humans. Porcine insulin is also structurally similar to human insulin and has been used safely for treating patients with type 1 diabetes for many years. Adult pig islets are often used however, adult pig islets are difficult to isolate and exhibit poor growth potential. Recently, Korbutt *et al.* (9) developed a simple procedure for isolating abundant islets from neonatal pigs. These islets were shown to have growth potential both *in vitro* and *in vivo*.

The expression of Galα(1,3)Gal on porcine islet cells remains controversial. Several studies have shown that Galα(1,3)Gal is not expressed on porcine islet endocrine cells (10-13), while studies performed in Chapter II demonstrated that approximately 30% of NPI cells expressed Galα(1,3)Gal and this expression was also detected on endocrine cells (14). The discrepancy between these studies is not clear, however, it is possible that Galα(1,3)Gal expression on porcine islet cells may be age-dependent. In this study we further examined the expression of Galα(1,3)Gal on non-matured, *in vitro* and *in vivo* matured NPI as well as adult pig islets and determined if Galα(1,3)Gal expression is age-dependent. In addition, the growth characteristics of NPI are not completely understood. It is generally believed that during fetal and neonatal life, islets of Langerhans are newly formed from precursor cells that bud off from the pancreatic ductules (15-17). The existence of such precursor cells in the ductal epithelium is supported by the morphological observation that fetal and neonatal islets are often closely associated with ductal epithelium. Cytokeratin (CK) proteins are phenotypic markers that could be used to trace the differentiation of ductal to islet cells. These proteins constitute the intermediate filaments of the cytoskeleton in epithelial cells and are expressed in a tissue type-specific manner, distinct subsets being expressed in different epithelial tissues (17, 18). In human pancreas, CK7 has been identified as the typical intermediate filament components of ductal epithelium and therefore will be used in this study as a marker for NPI cytodifferentiation and growth.

III-B) MATERIALS AND METHODS

1. Preparation of Neonatal Porcine and Adult Pig Islets

Isolation of NPI have been previously described (9). Briefly, Landrace-Yorkshire neonatal pigs ages 1-3 days (1.5-2.0 kg body wt) of either sex were anesthetized with Halothane and subjected to laparotomy and exsanguination. The pancreases were removed, cut into small pieces, and digested with 2.5 mg/ml collagenase (Sigma, St. Louis, MO). After filtration through a nylon screen (500 μm), the tissue was cultured for 9 days in HAM's F10 medium (Gibco, Burlington, Canada) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (Fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, U.K.), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Isolation of adult pig islets was previously described (19). Briefly, miniature swine Troll (age 18-19 months) were totally pancreatectomized under general anaesthesia and pancreata were intraductally distended with UW-solution (Du Pont, Bad Homburg, Germany) containing 0.4% (w/v) collagenase (Serva, Heidelberg, Germany) and digested in a continuous digestion-filtration device at 32 °C. Free islets were separated from non-islet tissue utilizing a Ficoll-Na-diatrizoate density gradient on a Cobe 2991 cell separator. Purified islets were cultured at 37 °C for 24 hours then dissociated into single cells.

2. *In Vitro* Maturation of NPI

After 9 days of culture, non-matured NPI were washed with Hank's balanced salt solution (HBSS) supplemented with 10 mmol/l HEPES. Islets were then resuspended in 0.44 ml HBSS and 0.55 ml of 1.5% (w/v) highly purified alginate (Metabolex, Inc., Hayward, CA) dissolved in HBSS. The resulting islet/alginate mixture was vortexed to obtain a homogeneous solution, then transferred into a 1.0 ml syringe. Microcapsules (250-350 μm in diameter) were formed by passing the alginate /islet suspension through an electrostatic generator followed by collection in a 120 mmol/l $CaCl_2$ (10 mmol/l HEPES, 0.01% Tween 20) solution for 10 minutes. The capsules were washed by gravity sedimentation in supplemented HAM's F10 medium and cultured in the same medium with autologous pig serum for 7 days at 37 °C. After culture, alginate capsules were dissolved by incubating encapsulated islets in calcium-free medium for 15 minutes at 37 °C. The naked islets were washed with calcium-free medium three times and dissociated into single cells as described below.

3. Preparation of NPI and Adult Pig Islet Cells

After culture, NPI and adult porcine islets were dissociated into single cells by gentle agitation in calcium-free medium containing 15 μg/ml trypsin (Boehringer Mannheim, Laval, Canada) and 4 μg/ml DNAse (Boehringer Mannheim). The cell suspension was filtered through a 63 μm nylon screen to remove cell clumps and then centrifuged through Percoll (Sigma) of 1.040g/ml density to eliminate dead cells and debris. Cells were then washed with phosphate-buffered saline (PBS) three times and kept either in suspension for FACS analysis (in the case of non-matured NPI cells) or distributed over adhesion microscope slides (FGR Steinmetz Inc., Surrey, Canada) for immunohistohistochemical analysis.

4. *In Vivo* Maturation of NPI

Non-matured NPI were transplanted under the left kidney capsule of Halothane-anesthetized Scid mice (6-8 weeks old; Taconic Farms Inc., Germantown, NY). Aliquots of 2,000 NPI were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the kidney capsule with the aid of a micromanipulator syringe. Once the tubing was removed, the hole in the renal capsule was sealed using a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL). The animals were returned to their cages and islet grafts were allowed to establish at the transplant site. Nephrectomy of the graft-bearing kidney was performed at specified time interval post-transplantation. Tissues were then processed for immunohistochemical analysis to determine Galα(1,3)Gal and CK7 expression as well as the presence of insulin-secreting cells in the grafts.

5. Immunocyto/histochemical Analysis

a) Single label immunocytochemical analysis

NPI and adult pig islet cells were fixed in Bouin's solution for 30 minutes, washed three times with 70% ethanol, and stored at 4 °C. Expression of Galα(1,3)Gal on NPI cells and grafts as well as on adult pig islet cells was detected using biotinylated Galα(1,3)Gal-specific BS-1 isolectin B4 from *Bandeiraea simplicifolia* (1:25 dilution;

Sigma). Cytokeratin expression was determined using the microwave antigen retrieval method where paraffin graft sections were first treated with 0.1M citrate buffer (3 times of 30 seconds each with a maximum strength of a domestic microwave) then digested with trypsin solution (0.1% trypsin and 0.125% $CaCl_2$) for 5 minutes. Samples were washed three times with PBS. For single islet cell preparation, cells were exposed to citrate buffer and microwave heat for 30 seconds (6 times of 5 seconds each). CK7 was detected using a monoclonal mouse anti-human CK7 antibody (1:200 dilution; Dako Diagnostics Canada Inc., Mississauga, Canada) and biotinylated anti-mouse IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA). Monoclonal mouse anti-porcine vimentin (1:1,000; Dako Diagnostics) was used to detect rapidly dividing cells within the NPI and adult pig islet cell preparations.

The presence of pancreatic hormones was determined using guinea pig anti-porcine insulin antibody (1:1,000 dilution; Dako Diagnostics) followed by biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories), rabbit anti-porcine glucagon antibody (1:1,000 dilution, Dako Diagnostics), rabbit anti-human somatostatin antibody (1:1,000 dilution; Dako Diagnostics), rabbit anti-human pancreatic polypeptide antibody (1:1,000 dilution; Dako Diagnostics) and biotinylated goat anti-rabbit IgG secondary antibody (1:200 dilution; Vector Laboratories). The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) method was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) to produce a brown color.

b) Double label immunohistochemical analysis

NPI grafts were fixed in Bouin's solution for 2 hours, washed three times in 70% ethanol and embedded in paraffin. Sections of 5 μm were first stained with either biotinylated Galα(1,3)Gal-specific lectin or mouse anti-human CK7 antibody for 30 minutes followed by appropriate biotinylated secondary antibody (for CK7 only as described above) for 20 minutes. The ABC/HP complex and DAB as chromagen were used to detect cells that express Galα(1,3)Gal or CK7. The same tissue sections were subsequently stained with guinea pig anti-porcine insulin antibody and biotinylated goat anti-guinea pig secondary antibody. The avidin-biotin complex/alkaline phosphatase (Vector Laboratories) method and Fast Red as chromagen (Vector Laboratories) were used to obtain a red reaction.

6. Fluorescence Activated Cell Sorter (FACS) Analysis

To further characterize the cellular composition of Galα(1,3)Gal-positive and negative NPI cells, non-matured NPI cells were first stained with fluorescein isothiocyanate (FITC)-conjugated BS-1 isolectin B4 (1:50 dilution; Sigma) for 1 hour on ice, washed three times, and submitted to FACS analysis (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL) for the purification of Galα(1,3)Gal-positive and -negative islet cells. The fluorescence emitted by FITC-labeled cells was selected as the sorting parameter. Positive controls for this experiment included porcine aortic endothelial cells (PAEC) stained with FITC-conjugated IB4 lectin using the same protocol for NPI cells. The efficacy of the cell separation was evaluated by visually comparing the fluorescence of sorted Galα(1,3)Gal-positive and -negative cells, as well as by re-analyzing each population by flow cytometry to detect the relative percentage of Galα(1,3)Gal-positive cells in each population. Sorted cells were then stained for CK7 and/or insulin as described above.

7. Statistical Analysis

Data are expressed as means±SEM of n independent observations. Significant differences among experimental groups were tested using analysis of variance (ANOVA) and differences between two groups were tested using paired T-test. $p < 0.05$ was considered to be statistically significant.

III-C) RESULTS

1. Cellular Composition of Non-Mature, *In Vitro* Mature NPIs, and Adult Pig Islets

One-color immunohistochemistry staining (Table 3-1) revealed that significantly more cells in the non-matured NPI expressed Galα(1,3)Gal (19.7±3.3%) compared to *in vitro* matured NPI (11.0±2.8%) and adult pig islets (5.1±1.3%). Similar pattern of distribution was also observed for CK7 and vimentin. Vimentin is a marker for a wide variety of mesenchymal cells. Expression of vimentin by epithelial cells is commonly seen in rapidly growing tissue in culture or regeneration. Non-matured NPI consist of 28.0±4.2% CK7-positive cells compared to 10.5±1.8% of *in vitro* matured NPI and 1.3±0.5% of adult pig islets. Vimentin-positive cells in non-matured NPI (15.0±3.9%) was approximately 2-fold (7.6±1.3%) and 10-fold (1.4±0.5%) higher than in *in vitro* matured NPI and adult pig islets, respectively which suggest that cells in the NPI are rapidly dividing. The proportions of glucagon, PP, and somatostatin were similar to the patterns observed for Galα(1,3)Gal, CK7, and vimentin except that there was a slight increase in the number of cells stained positive for glucagon and PP in the *in vitro* matured NPI. This increase however, was not significant (Table 3-1). In contrast, significantly less insulin-positive cells in non-matured NPI (33.7±3.8%) was observed compared to *in vitro* matured NPI (42.1±3.1%) and adult pig islets (82.8±1.2%).

2. Distribution of CK7 and Insulin in Galα(1,3)Gal-Positive and -Negative NPI Cells

We have previously reported that approximately 30% of non-mature NPI cells express Galα(1,3)Gal and 70% did not (14). After FACS analysis of non-mature NPI cells stained with FITC-conjugated IB4-lectin the distribution of CK7 and insulin in Galα(1,3)Gal-positive and -negative cell fractions was determined by immunocytochemical analysis. Significantly more CK7-positive cells (Table 3-2) were observed in Galα(1,3)Gal-positive population (24.4±10.4%) compared to Galα(1,3)Gal-negative cell fraction(1.8±0.7%). In contrast, there were significantly more insulin-positive cells in Galα(1,3)Gal-negative cell population than Galα(1,3)Gal-positive cell fraction (32.4±3.8% vs 11.7±2.1%, respectively). We found no significant difference however, in the number of cells stained positive for both CK7 and insulin in both Galα(1,3)Gal-positive and -negative populations (0.7±0.2% vs 1.4±0.9%).

3. *In Vivo* Expression of Galα(1,3)Gal and CK7

The expression of Galα(1,3)Gal and CK7 on NPI grafts were examined at several time intervals post-transplantation. The distribution pattern of Galα(1,3)Gal staining was most intense on day 1 post-transplantation (Figure 3-1A). IB4 lectin reacted strongly with ductal epithelium and some centrally located islet cells. Similar intensity of immunostaining for Galα(1,3)Gal was observed on day 7 (Figure 3-1B) however, the epithelial ducts can now be seen as branched structures located on the upper and bottom portion of the islet graft. There were also Galα(1,3)Gal-positive cells present in the middle of the graft. These observations were also evident on day 30 post-transplantation except there were less Galα(1,3)Gal-positive cells seen in the middle part of the graft (Figure 3-1C) and epithelial ducts appear to have migrated towards the center of the graft. On day 45 post-transplantation a faint staining for Galα(1,3)Gal was seen within the insulin-positive islets while intense staining was seen in majority of the vascular structures (some surrounding the islets and some scattered in the graft) (Figure 3-1D). At this time more insulin-positive cells were observed. On day >200 post-transplantation, very few cells (restricted to mouse endothelium originating from revascularization of the grafts) expressed Galα(1,3)Gal while majority of the cells within the graft were strongly stained positive for insulin (Figure 3-1E).

Anti-CK7 antibody also reacted strongly to ductal epithelium (Figure 3-2). On day 1 post-transplantation the ductal epithelia were present as branched structures, while the insulin-positive cells were arranged as short lines of single cells or small clusters of cells closely attached to the ductal epithelium (Figure 3-2A). On day 7 post-transplantation, the epithelial ducts consisted of one layer of cells forming a round structure or a network of round and elongated structures with some insulin-positive cells intermingled with CK7-positive duct cells (Figure 3-2B). On day 30 post-transplantation, more insulin positive cells are found within the graft and majority of these cells do not express CK7 (Figure 3-2C). Clearly around this time, large clusters of insulin-positive cells appear to be budding off from CK7-positive epithelial ducts. Some of the insulin-positive cell clusters were still closely attached to the ductal epithelia and majority of these ducts are located in the middle part of the graft. On day 45 post-transplantation, very few CK7-positive ducts were observed as more insulin-positive cells were formed (Figure 3-2D) and on day >200 post-transplantation the graft consisted mainly of insulin-positive cells (Figure 3-2E).

Table 3-1. Cellular composition of non matured, *in vitro* matured NPI and adult pig islets.

	Percentage of total cells						
Islets	αGal^a	Vimentin^a	CK7^a	Pancreatic Polypeptide^b	Insulin^a	Glucagon^b	Somatostatin^a
Non-matured NPI	19.7±3.3 (14)	15.0±3.9 (12)	28.0±4.2 (14)	8.7±1.7 (12)	33.7±3.8 (13)	19.5±3.0 (13)	8.2±1.0 (14)
In vitro matured NPI	11.0±2.8 (7)	7.6±1.3 (7)	10.5±1.8 (9)	10.4±0.9 (9)	42.1±3.1 (9)	23.3±1.4 (9)	8.0±0.9 (9)
Adult	5.1±1.3 (6)	1.4±0.5 (4)	1.3±0.5 (6)	4.5±1.3 (4)	82.8±1.2 (6)	9.7±3.2 (6)	4.0±1.0 (6)

Islets were dissociated into single cells and fixed with Bouin's solution. Cells were then stained with appropriate antibodies and positive reaction was detected by HP/ABC. (n) represents the number of samples in each case. ^{a}p<0.01 ; ^{b}p<0.05 by ANOVA.

Table 3-2. Expression of CK7 and/or insulin in Galα(1,3)Gal-positive and -negative non-mature NPI cells.

		Percentage of total cells		
Cell Population	n	CK7^a	Insulin^b	CK7/Insulin
Galα(1,3)Gal-positive	7	24.4±10.4	11.7±2.1	0.7±0.2
Galα(1,3)Gal-negative	9	1.8±0.7	32.4±3.8	1.4±0.9

Gal-positive and -negative cells were fixed with Bouin's and stained for CK7 and/or insulin. Positive reaction was detected using the ABC/HP and /or AP/ABC. ^{a} p<0.003 ;
^{b} p<0.005 by Mann-Whitney U Rank Sum Test.

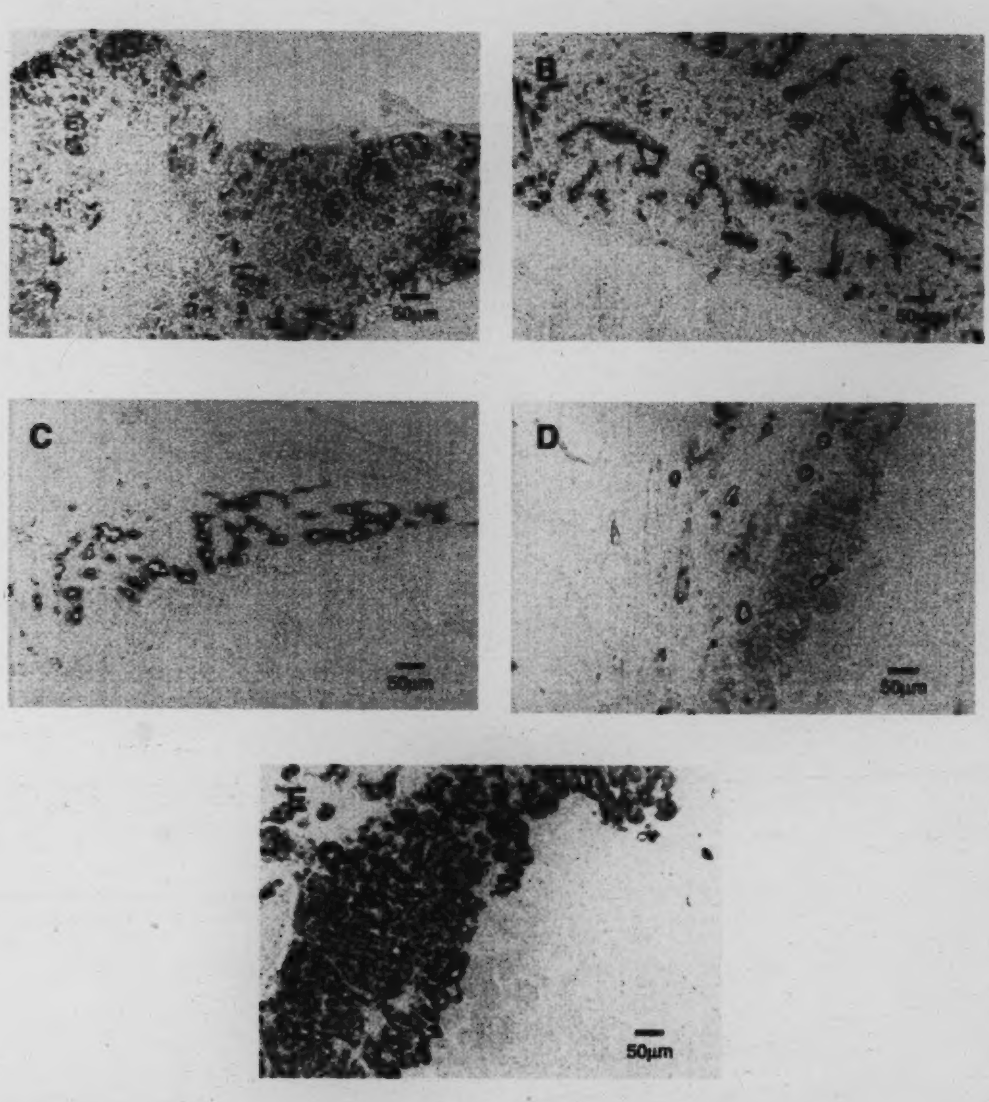

Figure 3-1. Expression of Galα(1,3)Gal and insulin on NPI xenografts. Expression of Galα(1,3)Gal was detected on NPI grafts harvested on days 1 (A), 7 (B), 30 (C), 45 (D) and >200 (E) post-transplantation. Galα(1,3)Gal (brown) was detected using biotinylated IB4 lectin and insulin-positive cells (red) were detected using anti-porcine insulin antibody. On days 1 and 7 more cells in the graft express Galα(1,3)Gal, then decreased on days 45 and >200 where majority of the cells are insulin-positive.

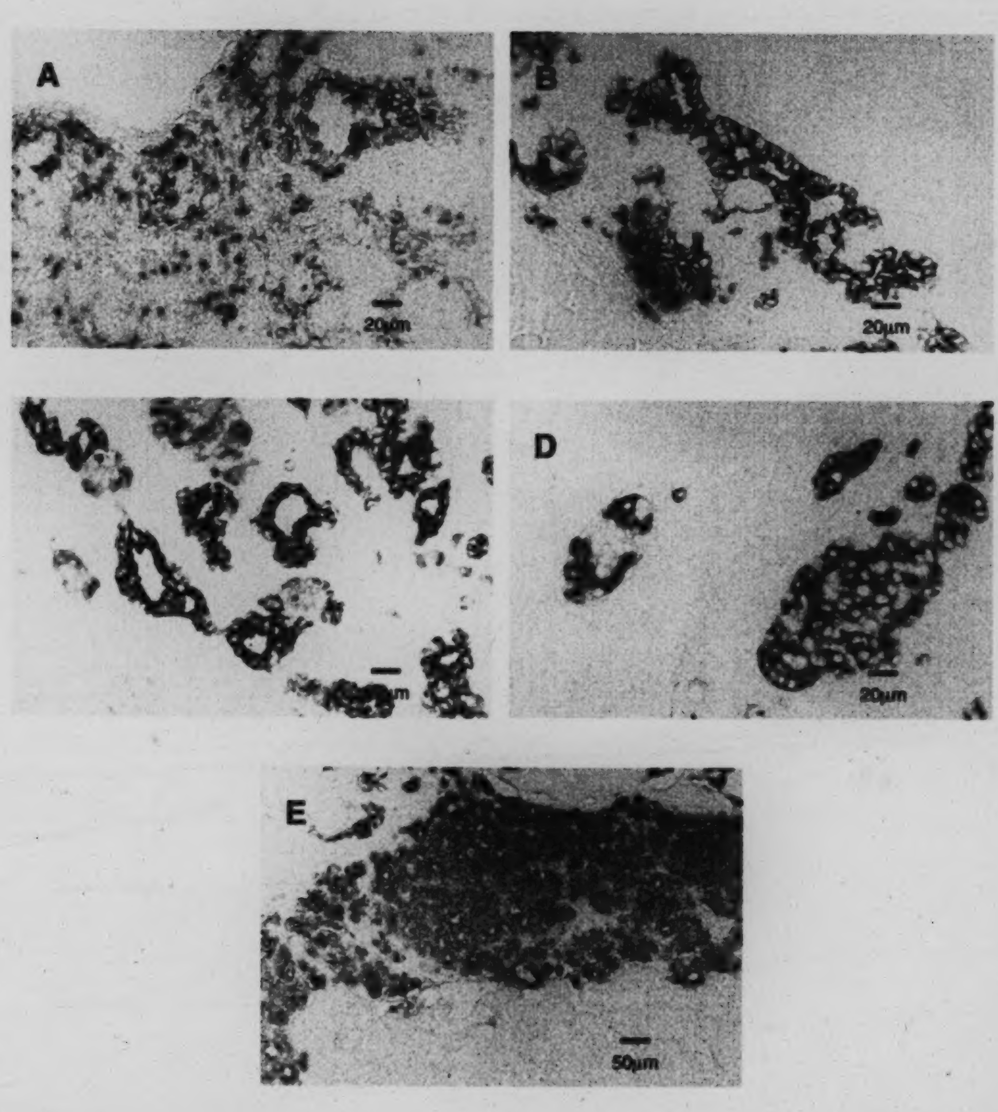

Figure 3-2. Expression of CK7 and insulin on NPI xenografts. Grafts were harvested on days 1 (A), 7, (B), 30 (C), 45 (D) and >200 (E) post-transplantation. CK7-positive epithelial ducts (brown) are detected using anti-human CK7 antibody and insulin-positive cells (red) are stained with anti-porcine insulin antibody. On day 30 (C), clusters of insulin-positive cells appear to be budding off from the epithelial ducts.

III-D) DISCUSSION

In this study we demonstrate both *in vitro* and *in vivo* that Galα(1,3)Gal expression on porcine islet cells is age-dependent. We found that Galα(1,3)Gal was highly expressed in young and developing NPI cells (as characterized by expression of CK7) and decreases when these cells have differentiated into functionally mature insulin-producing β cells. It has been shown that during development certain antigens are expressed at some stages only to disappear as the cells mature (20) and Galα(1,3)Gal may be one of these antigens. The function(s) of many oligosaccharides present on glycolipids and glycoproteins is not known due to its complicated pathways that have evolved to synthesize them. There has been suggestions that oligosaccharides on cell-surface proteins provided the cell with a protective coat that allowed the cell freedom to change shape and move. This activity is more foreseeable in developing islet cells than in mature ones. Since we have shown in neonatal pig pancreas that insulin-positive β cells were first randomly scattered as single cells or small clusters of cells frequently arranged alongside or within the duct lining (14). Further culture of the neonatal pancreatic tissue for 9 days resulted in the formation of spherical structures containing insulin-positive cells similar to adult pig islets. Galα(1,3)Gal and other oligosacchirides may be responsible for the formation of spherical structure and migration of endocrine cells to form an islet. It is also possible that Galα(1,3)Gal and other oligosccharides may play a role in cell-to-cell communication within the islet. It is known that individual islets present a typical topographical distribution of different cell types with a central bulk of insulin-containing β cells, a peripheral rim of α cells, PP-secreting cells and intermediate placed δ cells (21).

However, it is not known how this cellular arrangement is accomplished. Another possible function of Galα(1,3)Gal is to provide protection from harsh conditions surrounding the islet cells. For example, glycolipids on the plasma membrane of epithelial cells are confined to the apical surface where they may help to protect the membrane from low pH and degradative enzymes frequently present in the pancreas.

The mechanisms underlying NPI cell differentiation are still poorly understood. It is generally believed that, in embryos, the pancreatic duct contains undifferentiated precursor cells that migrate away from the duct to form islet and acinar cells (22). Endocrine cell differentiation occurs as soon as the pancreatic cell precursors emerge from the pancreatic duct and cell-specific expression is regulated at the transcription level by transacting proteins (such as Pdx1 and Nkx2.2), which bind to short DNA sequences located upstream from the transcription initiation site (23, 24). In the neonatal rat, Bouwens *et al.* (17) showed that the expression of the ductal CKs in islets and the observed continuity of islets with ductular epithelium are strongly suggestive for the differentiation of ductular cells to islet endocrine cells. In this study, we have shown that NPI contain a high proportion of precursor duct-like cells that differentiate into insulin-producing β cells. The presence of more CK7-positive cells in non-matured NPI compared to *in vitro* matured NPI and adult pig islet cells indicates the existence of transitional cytodifferentiation forms. Furthermore, the number of vimentin-positive cells is also higher in non-mature NPI than those seen in *in vitro* matured NPI and adult pig islets. culture or regeneration. In addition to differentiation of ductal cell precursor, it is also possible that pre-existing non-β endocrine cells (*i.e.* α, δ and PP-secreting cells) may differentiate into β cells to meet the functional demand and maintain euglycemia. In the whole human fetal pancreas a parallelism between the increase in β cell volume and in total glandular tissue has been observed (25), while the relative α cell volume reached a peak at a fetal age of 17-19 weeks and was followed by a marked decrease until the start of neonatal life. It was also observed that the δ cells reached their highest volume density in the neonatal period (26, 27). This may explain in part the lower number of non-β endocrine cells in the adult pig islets compared to non-matured and *in vitro* matured NPI. It has been the assumption that islet cells co-expressed two or more hormones during development (22). These mixed cells probably represent precursors which would differentiate into mature cells expressing a single specific product (22). In mouse, double label immunohistochemical studies revealed that all cells which expressed insulin also contained glucagon. (22, 28). Cells containing both insulin and glucagon were also detected in human and porcine fetal pancreas at the electron-microscopic level. Islet growth can also occur by replication of pre-existing β cells. Replication has been thought to be the principal means of expansion after birth, but with a capacity that diminishes with age (29). Preliminary data in our laboratory indicates that approximately 13% of NPI cells proliferate as measured by incorporation of BrdU (data not shown).

Taken together, these results demonstrate that expression of Galα(1,3)Gal on porcine islet cells is age-dependent and may explain in part why in other studies this antigen was not detected on endocrine cells. This results also suggest that adult pig islet cells may be less susceptible to killing by complement. However, when we treated the adult pig islet cells with rabbit complement, these cells were also killed (data not shown).

These further support our findings in Chapter II that Galα(1,3)Gal may not be critical in the lysis of porcine islet cells and that hyperacute rejection of this tissue can be mediated by complement alone. The factors involved in islet growth are only partially defined and whether Galα(1,3)Gal plays a role in the development and maturation of islets remains to be determined. A better understanding of β cell or islet growth may lead to new therapies for the treatment of diabetes and the ability to amplify islet tissue *in vitro* would facilitate islet transplantation as a therapy (29).

III-E) REFERENCES

1. Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabetes mellitus - from bench to bedside. Exp Clin Endocrinol 1995; 103: 143-159.

2. Warnock GL, Kneteman NM, Ryan EA, *et al.* Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 89-95.

3. Ricordi C, Tzakis AG, Carrol PB, *et al.* Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992; 53: 407-414.

4. Gores PF, Najarian JS, Stephanian E, *et al.* Insulin independence in type 1 diabetes after transplantation of purified islets from single donor with 15-deoxyspergualin. Lancet 1993; 341: 19-21.

5. Alejandro R, Lehmann R, Ricordi C, *et al.* Long-term function (6 years) of islet allografts in type 1 diabetes. Diabetes 1997; 46: 1983-1989.

6. Secchi A, Socci C, Maffi P, *et al.* Islet transplantation in IDDM patients. Diabetologia 1997; 40: 225-231.

7. Jaeger C, Brendel MD, Hering BJ, *et al.* Progressive islet graft failure occurs significantly earlier in autoantibody-positive than in autoantibody-negative IDDM recipients of intrahepatic islet allografts. Diabetes 1997; 46: 1907-1910.

8. Rabkin JM, Leone JP, Sutherland DER, *et al.* Transcontinental shipping of pancreatic islets for autotransplantation after total pancreatectomy. Pancreas 1997; 15: 416-419.

9. Korbutt GS, Elliott JF, Ao Z, *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-2129.

10. McKenzie IFC, Xing P-X, Vaughan HA, *et al.* Distribution of the major xenoantigen (gal(α1-3)gal) for pig to human xenografts. Transplant Immunol 1994; 2: 81-86.

11. McKenzie IFC, Koulmanda M, Mandel TE, *et al.* Pig-to-human xenotransplantation: The expression of Galα(1-3)Gal epitopes on pig islet cells. Xenotrnasplantation 1995; 2: 1-7.

12. McKenzie IFC, Koulmanda M, Mandel T, Sandrin MS. Expression of Galα(1,3)Gal by porcine islet cells and its relevance to xenotransplantation. Xenotrnasplantation 1995; 2: 139-142.

13. Oriol R, Ye Y, Koren E, Cooper DKC. Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433-1442.

14. Rayat GR, Rajotte RV, Elliott JF, Korbutt GS. Expression of Galα(1,3)Gal on neonatal porcine islet β-cells and susceptibility to human antibody/complement lysis. Diabetes 1998; 47: 1406-1411.

15. Hellerstrom C. The life story of the pancreatic B cell. Diabetologia 1984; 26: 393-400.

16. Swenne I. Pancreatic beta-cell growth and diabetes mellitus. Diabetologia 1992; 35: 193-201.

17. Bouwens L, Wang R-N, De Blay E, *et al.* Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas. Diabetes 1994; 43: 1279-1283.

18. Moll R, Franke WW, Schiller DL. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982; 31: 11-24.

19. Hering BJ, Mellert J, Brandhorst D, *et al.* Successful autotransplantation of purified porcine islets. Xenotransplantation 1995; 2: 193-196.

20. Kukowska-Latallo JF, Larsen RD, Nair RP, Lowe JB. A cloned human cDNA determines expression of a mouse stage-specific embryonic antigen and the Lewis blood group α(1,3/1,4)fucosyltransferase. Genes Dev 1990; 4: 1288.

21. Vinik A, Pittenger G, Rafaeloff R, Rosenberg L. Factors controlling pancreatic islet neogenesis. Tumor Biol 1993; 14: 184-200.

22. Teitelman G. On the origin of pancreatic endocrine cells, proliferation and neoplastic transformation. Tumor Biol 1993; 14: 167-173.

23. Darnell JE, Jr. Variety in the level of gene control in eucaryotic cells. Nature 1982; 297: 362-371.

24. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, *et al.* Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β cells. Development 1998; 125: 2213-2221.

25. Stefan Y, Grasso S, Perrelet A, Orci L. Quantitaive immunofluorescent study of the endocrine cell population in the developing human pancreas. Diabetes 1983; 32: 293-307.

26. Clark A, Grant AM. Quantitative morphology of endocrine cells in human fetal pancreas. Diabetologia 1983; 25: 31-35.

27. Rahier J, Wallon J, Henquin JC. Abundance of somatostatin cells in the human neonatal pancreas. Diabetologia 1980; 251-254.

28. Alpert S, Hanahan D, Teitelman G. Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 1988; 53: 295-308.

29. Bonner-Weir S, Smith FE. Islet cell growth and the growth factors involved. Trends in Endocrinol Metab 1994; 5: 60-64.

CHAPTER IV

MICROENCAPSULATION OF NEONATAL PORCINE ISLETS: PROTECTION FROM HUMAN ANTIBODY/COMPLEMENT-MEDIATED CYTOLYSIS *IN VITRO* AND LONG-TERM REVERSAL OF DIABETES IN NUDE MICE[1]

IV-A) INTRODUCTION

Islet transplantation is an attractive alternative treatment for patients with type 1 diabetes (1-7). There are however, currently two major barriers to overcome before islet transplantation can be offered as a therapy for diabetic patients. One is the poor availability of human pancreatic tissue and the other is the need for permanent immunosuppression to prevent rejection. In respect to the former, pigs are an attractive source of islets because they breed rapidly, have large litters, and porcine insulin has been used to treat patients with type 1 diabetes for many years. Recently, Korbutt *et al.* (8) developed a simple and reliable method to efficiently isolate large numbers of islets from neonatal pigs. These islets are easily maintained in tissue culture, correct diabetes in nude mice and have growth potential both *in vitro* and *in vivo* (8, 9). Although these characteristics make neonatal porcine islets (NPI) a very attractive source of insulin-producing tissue for clinical transplantation, we recently demonstrated that NPI are susceptible to lysis mediated by human antibody and complement *in vitro* (10, 11). One microencapsulation enhances survival of dog islets both *in vitro* and *in vivo* (12, 13) as well as partially protect NPI xenografts after implantation into diabetic NOD mice (14). It remains unknown however if microencapsulation can protect NPI from injury caused by human antibody and complement or if these islets can survive and correct diabetes long-term. The aims of this study were to examine the capacity of microencapsulation in protecting NPI from injury induced by human antibody and complement *in vitro* and to assess their ability in reversing hyperglycemia in diabetic nude mice.

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[1] *A version of this chapter has been submitted for publication. Rayat et al. 1999. Transplantation.*

IV-B) MATERIALS AND METHODS

1. Animals

One to three day-old Landrace-Yorkshire neonatal pigs (1.5 to 2.0 kg body weight) of either sex were used as islet donors. Male, inbred, athymic nude Balb/c mice (age 6 to 8 weeks) were used as recipients of NPI (the Jackson Laboratories, Bar Harbor, ME). Mice were rendered diabetic by intravenous injection of 90 mg/kg body weight alloxan (Sigma Chemical Co., St. Louis, MO; freshly dissolved in 1 mmol/l hydrochloric acid) 4 to 5 days before transplantation. All recipients in the study had blood glucose levels above 20 mmol/l. Blood samples were obtained from the tail vein to monitor glucose level (Medisense glucose meter; Medisense Canada, Mississauga, ON). Animals were maintained under virus-antigen-free conditioned rooms with free access to sterilized tap water and pelleted food.

2. Preparation and Encapsulation of NPI

The method used to isolate NPI has been previously described (8). Briefly, neonatal pigs of either sex were anesthetized with Halothane and subjected to laparotomy and exsanguination. The pancreas was removed, cut into small pieces, and digested with 2.5 mg/ml collagenase (Sigma). After filtration through a nylon screen (500 μm), the tissue was cultured for 7 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10 mmol/l glucose, 50 μmol/l isobutylmthylxanthine (ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (BSA; fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C (5% CO_2, 95% air).

After culture, NPI were washed with Hank's balanced salt solution (HBSS) supplemented with 10 mmol/l HEPES. Islets were then resuspended in 0.44 ml HBSS and 0.55 ml of 1.5% (w/v) highly purified alginate (Metabolex, Inc., Hayward, CA) dissolved in HBSS. Microcapsules (250-350 μm) were formed by passing the alginate/islet suspension through an electrostatic generator followed by collection in a 120 mmol/l $CaCl_2$ (10 mmol/l HEPES, 0.01% Tween 20) solution for 10 minutes. The capsules were washed by gravity sedimentation in supplemented HAM's F10 medium and cultured in the same medium at 37 °C for two days. Controls included non-encapsulated islets which were also cultured in HAM's F10 for an additional 2 days.

3. *In Vitro* Human Antibody/Complement-Mediated Cytotoxicity Assay

The method for the *in vitro* cytotoxicity assay has been previously described (10, 11). Briefly, encapsulated and non-encapsulated NPI were incubated for 24 hours in either heat-inactivated (HI; complement-depleted) human AB serum or fresh human serum (containing complement) diluted 1:1 with HAM's F10 medium (supplemented as above). After the incubation period, NPI were recovered from the petri dishes, washed in HAM's F10 medium, and the preparations were assessed for recovery of cellular insulin and DNA contents as well as functional viability. Insulin content was measured after extraction in 2 mmol/l acetic acid containing 0.25% BSA (8). Samples were sonicated in acetic acid, centrifuged (800g, 15 minutes), then supernatants were collected and stored at -20 °C until assayed for insulin content by ELISA (Boehringer Mannheim, Laval, PQ). For DNA content, aliquots were washed in citrate buffer (150 mmol/l NaCl, 15 mmol/l citrate, 3 mmol/l EDTA, pH 7.4) and stored as cell pellets at -20 °C. Before the assay, cell pellets were placed in 450 µl of lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.5% Triton X-100, 4 °C, pH 7.5), sonicated, supplemented with 25 µl of Proteinase K solution (8 mg/ml), vortexed, and incubated at 65 and 70 °C for 45 and 10 minutes, respectively. Lysates were supplemented with 25 µl of RNAse A solution (10 mg/ml), vortexed and incubated for 1 hour at 37 °C. Aliquots of 25 and 50 µl were assayed in duplicate by diluting them in 1.0 ml of DNA buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.5) and measuring fluorescence at a wavelength of 490 nm (excitation) and 515 nm (emission) after addition of 1.0 ml Pico Green reagent (1/200 dilution with DNA buffer). Samples were run in parallel with and diluted in proportion to a seven-point (0-400 ng/ml) standard curve which was generated using calf thymus DNA.

For assessment of *in vitro* functional viability, the secretory response to glucose of non-encapsulated and encapsulated NPI was determined using a static incubation assay (8, 10). The cultured fractions were recovered from the petri dishes, washed, and aliquots of 50-100 NPI were incubated for 120 minutes in 1.5 ml of RPMI medium supplemented with 2 mmol/l L-glutamine, 0.5% BSA and either 2.8 mmol/l glucose, 20 mmol/l glucose or 20 mmol/l glucose plus 10 mmol/l theophylline. Tissue and medium were then separated by gravity sedimentation and assayed for their respective insulin contents. The insulin content of the medium was expressed as a percentage of the total content (*i.e.*, tissue plus medium). Stimulation indices were calculated by dividing the amount of insulin release at 20 mmol/l glucose (± theophylline) by that released at 2.8 mmol/l glucose.

4. Transplantation of Encapsulated NPI and Metabolic Follow-up

Non-encapsulated or encapsulated islets (2,000) were transplanted under the kidney capsule or intraperitoneally into alloxan-induced diabetic nude mice, respectively. Recipients were monitored for blood glucose levels once a week between 8:00 and 11:00 a.m. When the blood glucose level was ≤ 8.4 mmol/l, the transplant was deemed a success. At 40 weeks post-transplantation, an oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT) were performed on NPI recipients with normalized basal glycemia and in normal controls. After a 2-hour fast, D-glucose (3 mg/g body weight) was administered as a 50% solution into non-anesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 minutes. Five to seven days after the GTT's, capsules were recovered by an intraperitoneal lavage for dithizone staining or EM analysis (as described below). To confirm the efficacy of the encapsulated NPI at correcting diabetes, the pancreas of each recipient was assayed for insulin content as previously described (8). Similarly, animals with renal subcapsular grafts were nephrectomized and subsequently monitored to confirm a return to hyperglycemia.

5. Morphological assessment of non-encapsulated and encapsulated NPI pre-and post-transplantation

The morphology of encapsulated NPI (1,000) immediately prior to transplantation was examined by dithizone staining (0.02% final concentration), immunohistochemical staining for insulin (8, 11), and electron microscopy. Samples were fixed in Bouin's solution for 2 hours, washed three times with 70% ethanol, and embedded in paraffin. Next, 5µm sections were stained with guinea pig anti-porcine insulin antibody (1:1,000 dilution; Dako Laboratories, Mississauga, ON) for 30 minutes followed by biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA). For electron microscopic (EM) analysis, NPI were fixed in 2.5% (vol/vol) glutaraldehyde (Millonig's buffer, pH 7.2), post-fixed in 1.5% (wt/vol) OsO4, washed in distilled water, then dehydrated successively in 50, 70, 80, 90, and 100% ethanol before embedding in araldite. Sections were stained with lead citrate and uranyl acetate then subsequently examined in a Hitachi H 7000 (Hitachi Ltd., Tokyo, Japan) transmission electron microscope.

6. Statistical Analysis

Data are expressed as means±SEM of n independent observations. Statistical significance of differences among groups was determined using one-way analysis of variance (ANOVA) and between two groups using paired T-test. $p<0.05$ was considered statistically significant.

IV-C) RESULTS

1. Cytotoxicity of Human Serum and Complement

Incubation of non-encapsulated NPI for 24 hours in the presence of complement-containing fresh human serum resulted in a 53% loss of cellular insulin mass ($p<0.001$) and a 51% reduction in recoverable DNA content ($p<0.001$) when compared to non-encapsulated NPI cultured in complement-depleted HI human serum (Table 4-1). In contrast, exposure of encapsulated islets to fresh human serum had no cytotoxic effect on the islets (91.0±8.0% insulin and 94.0±8.0% DNA recovered). Similar results were observed when encapsulated islets were exposed to HI human serum (96.0±9.0% insulin and 89.0±3.0% DNA recovered). It is worth noting at this point that these results are comparable to what we found in Chapter II, where we showed that treatment of NPI cells with HI human serum did not result to significant lysis but resulted to >50.0% killing of the islets when exposed to fresh human serum as measured by neutral red staining (Table 2-2, Chapter II). However, in Chapter II, it appears that a very small percentage (2-3%) of NPI cells were killed after treatment with HI serum. We think that this is probably the result of several manipulations experienced by the cells during FACS analysis since cells treated with media alone exhibited similar results. In the present study, we used insulin and DNA recovery to assess viability of the islets since it is not feasible to assess the quantity of lysis in intact islets by neutral red.

Table 4-1. Effect of human serum and complement on non-encapsulated and encapsulated NPI.

Condition	Serum	n	Percentage Recovery Insulin	 DNA
Non-encapsulated	HI	5	100.0	100.0
	Fresh	5	47.0±6.0[a]	49.0±4.0[a]
Encapsulated	HI	5	96.0±9.0	89.0±3.0
	Fresh	5	91.0±8.0	94.0±8.0

Values are means±SEM of n independent experiments and are expressed as a percentage of the corresponding values for non-encapsulated NPI cultured in heat inactivated (HI) pooled human AB serum without complement. Statistical significance of differences between groups was calculated using one-way ANOVA. [a] p<0.001.

The secretory activity of NPI was tested by comparing the percentage of cellular insulin released at low glucose (2.8 mM), high glucose (20 mM), and high glucose plus 10 mM theophylline. Non-encapsulated NPI previously treated with complement deficient heat-inactivated human serum exhibited a mean stimulation index of 4.9±0.7 when compared to the release at high glucose *versus* at low glucose. When exposed to 20 mM glucose in combination with 10 mM theophylline, the stimulation index increased to more than 22-fold. On the other hand, insulin secretion of non-encapsulated islets exposed to human serum containing complement was markedly altered (Table 4-2).

Incubation at low glucose significantly ($p<0.05$) increased the secretory rate of non-encapsulated NPI pre-treated with fresh human serum; whereas insulin secretion was significantly reduced when exposed to high glucose plus theophylline. Thus the calculated stimulation indices after incubation with either 20 mM glucose or 20 mM glucose plus 10 mM theophylline were significantly lower ($p<0.001$ and $p<0.05$, respectively) compared to non-encapsulated NPI previously cultured with HI human serum. In contrast, no significant differences were observed in the insulin secretory activity between controls (non-encapsulated exposed to heat-inactivated serum) and microencapsulated NPI pre-treated with either fresh or heat-inactivated human serum. Thus the calculated stimulation indices of these groups were comparable.

Table 4-2. Effect of human serum and complement on the insulin secretory capacity of NPI.

			Isolated islet fraction (insulin secretory activity [% content])			Stimulation Indices	
Condition	Serum	n	2.8 mM glucose	20 mM glucose	20mM glucose + 10 mM theophylline	High:low	High + theophylline:low
Non-Encapsulated	HI	5	1.5±0.2	7.0±1.1	34.3±2.3	4.9±0.7	22.8±4.7
	Fresh	5	6.2±0.9^b	7.6±1.3	17.9±3.5^b	1.1±0.1^a	2.8±0.8^b
Encapsulated	HI	5	1.1±0.3	4.8±0.3	29.9±2.2	4.3±0.4	27.1±2.7
	Fresh	5	1.3±0.1	5.8±0.8	34.3±1.2	4.5±0.4	26.4±2.2

Values are means ± SEM of n independent experiments. In each experiment data were collected following culture in heat inactivated human serum (HI) or fresh human serum at 37 °C as described in Materials and Methods. Stimulation indices were calculated by dividing the amount of insulin released at high glucose (20 mmol/l) by that released at low glucose (2.8 mmol/l). Statistical significance of differences between groups was calculated by one-way analysis of variance. ^{a} $p<0.001$, ^{b} $p<0.05$.

2. Transplantation of NPI Into Diabetic Nude Mice

Figure 4-1A illustrates the blood glucose values during post-transplant follow-up period in the following groups of recipients: 1) non-encapsulated NPI transplanted under the kidney capsule, 2) non-encapsulated NPI implanted intraperitoneally, and 3) encapsulated NPI placed intraperitoneally. After alloxan administration, all recipients exhibited blood glucose values above 20 mmol/l. All animals transplanted intraperitoneally with encapsulated NPI exhibited blood glucose values ≤8.4 mmol/l within 8 weeks post-transplantation (Table 4-3, Figure 4-1A). This metabolic state was maintained over the 40 weeks follow-up period. Similar results were achieved when non-encapsulated NPI were implanted under the kidney capsule of diabetic nude mice (Table 4-3, Figure 4-1A). On the other hand, when non-encapsulated NPI were transplanted intraperitoneally, all recipients failed to achieve euglycemia and survived for only 21.0±4.0 days post-transplant.

Glucose tolerance tests were performed on normoglycemic recipients and controls at 40 weeks post-transplantation. Compared to age-matched normal control mice, animals implanted with encapsulated NPI (intraperitoneally) or non-encapsulated NPI (kidney capsule) showed significantly lower ($p<0.05$) glucose values at 15 and 30 minutes in both OGTT and IPGTT (Figure 4-1B and 4-1C). There was no significant difference in the glucose values at all time points between the two transplant groups. In all groups, the glucose values at 120 minutes post-glucose administration was not significantly different from the values at 0 minutes.

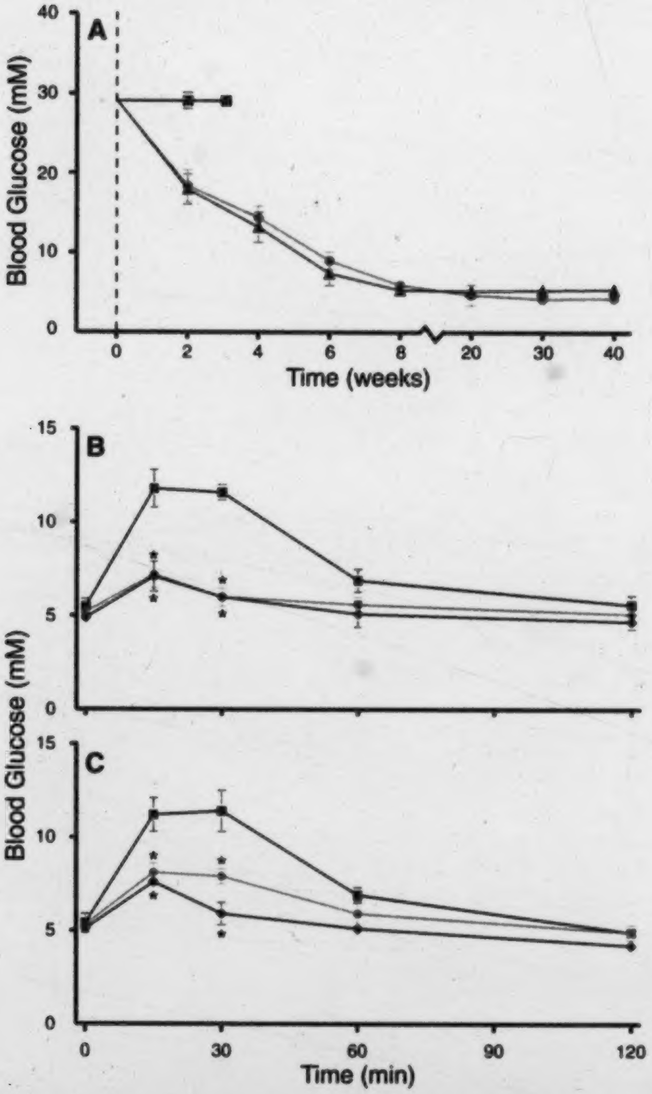

Figure 4-1. A) Blood glucose values in alloxan-induced diabetic nude mice transplanted with 2,000 NPI under the kidney capsule (non-encapsulated; ●; n=10) or intraperitoneally (encapsulated; ▲; n=16 or non-encapsulated; ■; n=6).
Blood glucose values during oral (B; OGTT); and intraperitoneal (C; IPGTT) administration of glucose to nude mice transplanted with non-encapsulated (◆; kidney; n=10) or encapsulated (●; intraperitoneally; n=12) NPI in comparison to age-matched normal control (■; n=6) mice at 40 weeks post-transplantation. Values are means ±SEM. Statistical significance of differences between groups was calculated by one-way analysis of variance. *p<0.05 *vs* normal controls.

Table 4-3. Metabolic follow-up of diabetic nude mice transplanted with 2,000 NPI.

Experimental Groups	Site	n	Weeks Post-transplantation							
			1	2	3	4	5	6	8	42
			Normoglycemic animals[a]/Total number of recipients							
Encapsulated	i.p.	16	0/16	0/16	4/16	8/16	12/16	14/16	16/16	16/16
Non-encapsulated	k.c.	10	0/10	0/10	3/10	5/10	7/10	9/10	10/10	10/10

Normoglycemia defined as blood glucose values ≤8.4 mmo/l. i.p., intraperitoneum; k.c., kidney capsule.

Examination of recovered encapsulated NPI at 42 weeks post-transplantation using dithizone revealed intact NPI with more intense dithizone staining compared to NPI before transplantation (Figure 4-2A, 4-2B). Similarly, immunohistochemical (Figure 4-2C, 4-2D) and electron microscopic (Figure 4-2E, 4-2F) analyses, showed fully differentiated islets containing numerous insulin-producing cells and well-granulated endocrine cells compared to islets before transplantation which contain less endocrine cells and more non-endocrine cells. Throughout the experimental period, the capsules remained intact with no signs of fibrosis observed on the capsules' exterior.

In all recipients of encapsulated (intraperitoneally) or non-encapsulated NPI (kidney capsule), removal of the islet grafts was followed by a rapid return to the diabetic state. The pancreatic insulin content of recipients of encapsulated NPI was <2% (<0.7 μg) of the insulin content of normal nude mice (38.0 μg; data not shown). These results demonstrate that the normoglycemia observed in the recipients is attributable to insulin production from grafts and not from residual pancreatic cells.

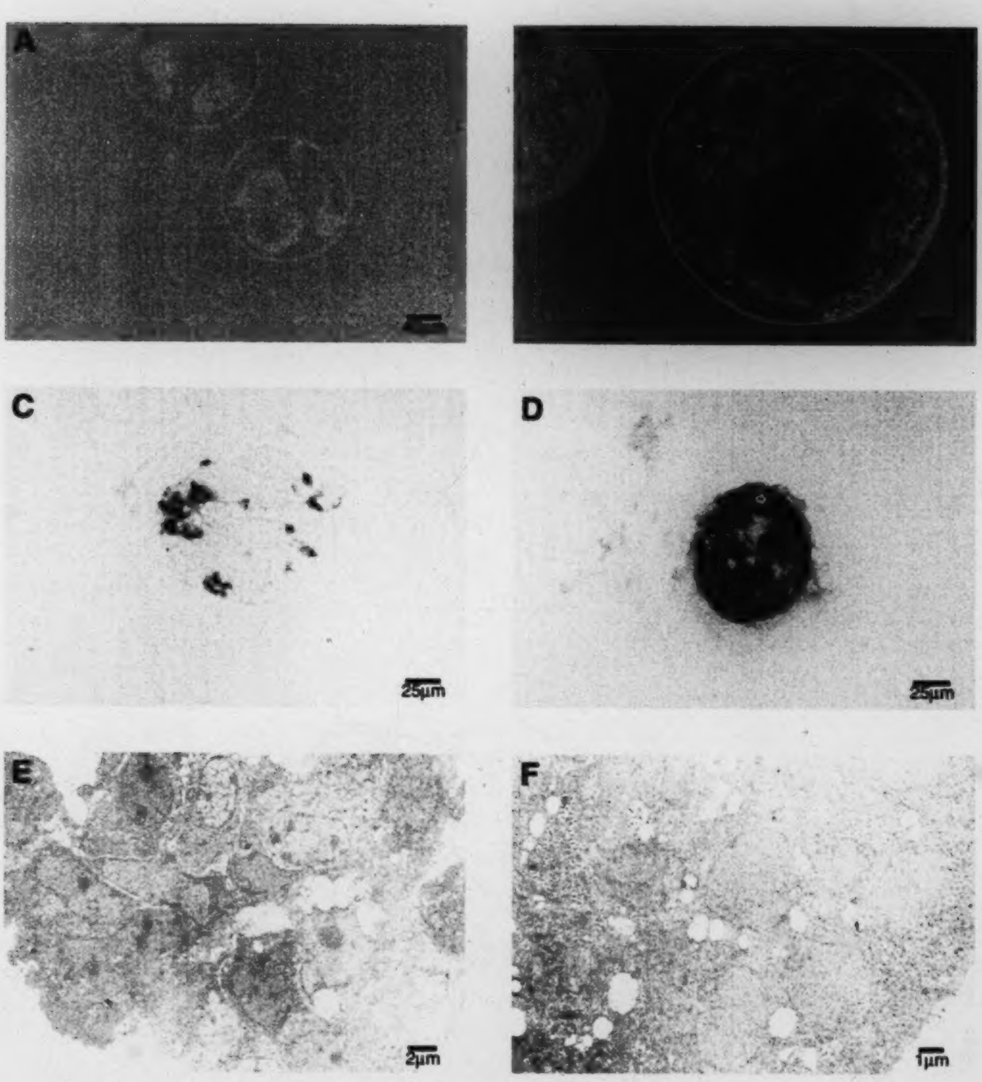

Figure 4-2. Morphology of encapsulated NPI before (A, C, E) and after (B, D, F) implantation to alloxan-induced diabetic nude mice. NPI were treated with dithizone (A,B), immunostained for the presence of insulin-positive cells (C, D), and processed for transmission electron microscopy (E, F).

IV-D) DISCUSSION

Neonatal porcine islets constitute an attractive source of insulin-producing tissue for clinical transplantation, however, several immunological obstacles must first be overcome. We have shown that human IgG and IgM natural xenoreactive antibodies bind to NPI cells (11), NPI cells express the Galα(1,3)Gal epitope (11), and NPI cells are susceptible to human antibody/complement-mediated cytolysis (10, 11). Taken together, one potential approach to prevent humoral-mediated destruction is to place the NPI grafts in an immunoisolation device to block access of human antibody and/or complement. In this study, we examined whether a highly purified alginate microcapsule can protect NPI from injury induced by human antibody and complement *in vitro*. The data presented here demonstrate that microencapsulated NPI are protected from the deleterious effects of fresh human serum containing complement since cellular insulin and DNA recoveries as well as insulin secretory activity were not adversely altered. In contrast, 24 hour exposure of non-encapsulated NPI to human serum and complement resulted in more than 50% loss of cellular insulin/DNA contents and an abnormal glucose responsiveness during an *in vitro* static incubation. The mechanism responsible for the protective effect of the alginate capsule is not known. We have however, determined that human IgM, but not IgG, is unable to diffuse across the alginate capsule used in the current study (data not shown). It is therefore possible that the alginate capsule blocks the passage of human IgM and more importantly large molecular weight products of complement activation and thereby, prevents destruction of the NPI. This concept is supported by the observation that human IgM as opposed to IgG is the predominant type of human anti-porcine xenoreactive antibodies (15-21) and that alginate-poly-L-lysine microencapsulated sensitized sheep erythrocytes were protected against activated complement fragments (22). Whether microencapsulation will also protect NPI from cell-mediated immune response remains to be determined. It may be more difficult to block this type of immune response since small antigens that can escape through the pores of the capsule might trigger a cellular immune response (most likely inflammatory) that will lead to destruction of the islets. Cytokines (for example IFN-γ and TNF-α) and various toxic products (for example nitric oxide) produced by immune cells attracted to the encapsulated islets may cause the death of the islets.

Diabetic nude mice were transplanted with encapsulated and non-encapsulated NPI in order to examine their efficacy at correcting diabetes in these animals. At week 8 post-transplantation, 100% of the mice receiving 2,000 encapsulated (intraperitoneally) or non-encapsulated (kidney capsule) NPI exhibited blood glucose levels ≤8.4 mmol/l. These data are comparable to our previous findings where 2,000 non-encapsulated NPI gradually achieved normoglycemia within 8 weeks following implantation under the kidney capsule of diabetic nude mice (8). Moreover, the present study demonstrates that encapsulated NPI are equally effective at correcting diabetes in nude mice when transplanted in the peritoneum. Furthermore, 2,000 non-encapsulated NPI were not able to reverse diabetes after intraperitoneal implantation. This was an expected result because it has been previously reported that intraperitoneal islet grafts are not very effective at correcting diabetes (23, 24). It is possible that our alginate microcapsule provides a support matrix for the NPI, thereby allowing them to survive and engraft in the peritoneal cavity.

Unexpected results were however observed during the glucose tolerance tests, because mice transplanted with encapsulated grafts not only exhibited comparable glucose tolerance to recipients of renal subcapsular NPI, but also had lower blood glucose levels at 15 and 30 minutes after glucose challenge when compared to normal controls. This is the first report demonstrating that microencapsulated islet grafts placed intraperitoneally can not only achieve long-term euglycemia but also glucose tolerance comparable to normal control animals. These data indicates that the alginate capsule is highly biocompatible thereby permitting optimal islet engraftment /survival, avoiding fibrotic reactions around the encapsulated islets, resulting in sufficient islet cell mass to achieve normoglycemia and glucose tolerance. Furthermore, the phenomenon that both transplant groups had lower blood glucose levels compared to normal controls likely results from different insulin secretory properties of porcine islets compared to mouse islets, as previously shown in our previous studies when porcine islets were implanted into diabetic nude mice (8).

Although NPI placed in either site were unable to achieve euglycemia immediately post-transplant, they eventually developed the ability to establish and maintain euglycemia during the 40 weeks follow-up period. At the time of transplantation, NPI were not fully matured/differentiated, thus it is likely that following transplantation they exhibited growth and/or differentiation of new ß cells until a critical mass was achieved to establish and maintain euglycemia. The morphological data indirectly support this concept, since at the time of transplantation NPI were composed of relatively few insulin-positive cells, whereas several weeks after implantation the recovered grafts were largely composed of β cells. These observations indicate that microencapsulated immature NPI grafts continue to grow and differentiate when transplanted into the peritoneum of diabetic nude mice.

In conclusion, microencapsulation protects NPI from the lysis mediated by human antibody and complement. Since previous studies (10, 11) showed that NPI are susceptible to human natural antibody/complement lysis *in vitro*, microencapsulation may be a strategy to prevent this form of humoral-mediated destruction. Furthermore, microencapsulation does not affect NPI survival and function following transplantation into the peritoneum of diabetic nude mice. Moreover, microencapsulated NPI grafts appear to mature and differentiate post-transplantation and they are not only capable of maintaining long-term euglycemia in diabetic nude mice, but can also achieve glucose tolerance. These results provide further information supporting the feasibility of using neonatal porcine islet transplantation as a treatment for diabetes in humans.

IV-E) REFERENCES

1. Warnock GL, Kneteman NM, Ryan EA, *et al.* Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 89-95.

2. Ricordi C, Tzakis AG, Carroll PB, *et al.* Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992; 53: 407-414.

3. Gores PF, Najarian JS, Stephanian E, *et al.* Insulin independence in type 1 diabetes after transplantation of unpurified islets from single donor with 15-deoxyspergualin. Lancet 1993; 341: 19-21.

4. Alejandro R, Lehmann R, Ricordi C, *et al.* Long-term function (6 years) of islet allografts in type 1 diabetes. Diabetes 1997; 46: 1983-1989.

5. Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabete mellitus - from bench to bedside. Exp Clin Endocrinol 1995; 103: 143-159.

6. Calafiore R. Perspectives in pancreatic and islet cell transplantation for the therapy of IDDM. Diabetes Care 1997; 20: 889-896.

7. Slover RH, Eisenbarth GS. Prevention of type I diabetes and recurrence of β-cell destruction of transplanted islets. Endocrine Reviews 1997; 18: 241-258.

8. Korbutt GS, Elliott JF, Ao Z, *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-2129.

9. Weber CJ, Hagler MK, Chryssochoos JT, *et al.* CTLA4-ig prolongs survival of microencapsulated neonatal porcine islet xenografts in diabetic NOD mice. Cell Transplantation 1997; 6: 505-508.

10. Korbutt GS, Aspeslet LJ, Rajotte RV, *et al.* Natural human antibody-mediated destruction of porcine neonatal islet cell grafts. Xenotransplantation 1996; 3: 207-216.

11. Rayat GR, Rajotte RV, Elliott JF, Korbutt GS. Expression of Galα(1,3)Gal on neonatal porcine islet β-cells and susceptibility to human antibody/complement lysis. Diabetes 1998; 47: 1406-1411.

12. Ao Z, Korbutt GS, Warnock GL, *et al.* Microencapsulation improves canine islet survival *in vivo*. Transplant Proc 1995; 27: 3349.

13. Ao Z, Korbutt GS, Warnock GL, *et al.* Microencapsulation enhances canine islet survival during long-term culture. Transplant Proc 1995; 27: 3350.

14. Ao Z, Suarez-Pinzon WL, Rajotte RV, *et al.* Transplantation of microencapsulated syngeneic and xenogeneic (neonatal porcine) islets in nonobese diabetic nude mice. Transplant Proc 1998; 30: 500.

15. Platt JL, Fischel RJ, Matas AJ, *et al.* Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. Transplantation 1991; 52: 214-220.

16. Platt JL, Lindman BJ, Geller RL, *et al.* The role of natural antibodies in the activation of xenogeneic endothelial cells. Transplantation 1991; 52: 1037-1043.

17. Xu H, Edwards N, Chen JM, *et al.* Identification and functional analysis of human natural anti-pig xenoantibodies. Transplant Proc 1994; 26: 1365.

18. Good H, Cooper DKC, Malcolm AJK, *et al.* Identification of carbohydrate structures which bind human anti-porcine antibodies: Implications for discordant grafting in man. Transplant Proc 1992; 24: 559-562.

19. Gambiez L, Salame E, Chereau C, *et al.* The role of natural IgM in the hyperacute rejection of discordant heart xenografts. Transplantation 1992; 54: 577-583.

20. Neethling FA, Koren E, Ye Y, *et al.* Protection of pig kidney (PK15) cells from the cytotoxic effect of anti-pig antibodies by α-galactosyl oligosaccharides. Transplantation 1994; 57: 959-963.

21. Vaughan H, Loveland BE, Sandrin MS. Galα(1,3)Gal is the major xenoepitope expressed on pig endothelial cells recognized by naturally occurring cytotoxic human antibodies. Transplantation 1994; 58: 879-882.

22. Darquy S, Pueyo ME, Capron F, Reach G. Complement activation by alginate-polylysine microcapsules used for islet transplantation. Artificial Organs 1994; 18: 898-903.

23. Fritschy WM, Van Straaten JFM, De Vos P, *et al.* The efficacy of intraperitoneal pancreatic islet isografts in the reversal of diabetes in rats. Transplantation 1991; 52: 777-783.

24. Davalli AM, Ogawa Y, Scaglia L, *et al.* Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes 1995; 44: 104-111.

CHAPTER V

NEONATAL PORCINE ISLET CELLS INDUCE HUMAN CD4+ BUT NOT CD8+ LYMPHOCYTE PROLIFERATION AND RESIST CELL-MEDIATED CYTOLYTIC INJURY *IN VITRO*[1]

V-A) INTRODUCTION

The severe shortage of human organ donors has stimulated extensive interest in xenotransplantation as an alternative to human allotransplantation. Pigs are presently considered the most likely source of organs since their organs have similar physiology and size to human organs. In addition, pigs breed in large numbers and porcine insulin has been used for many years to treat patients with diabetes. Recently, Korbutt *et al.* (1) developed a method of efficient isolation and *in vitro* culture of neonatal porcine islets (NPI). These islets contain a high proportion of insulin-positive cells which are capable of correcting hyperglycemia in nude mice. Thus, transplantation of NPI to reverse hyperglycemia in humans is an attractive alternative treatment because NPI offer the potential for unlimited supply of islets.

The presence in primate sera of preformed xenoreactive antibodies, primarily directed against Galα(1,3)Gal leads to complement activation and hyperacute rejection (HAR) of porcine xenografts within minutes to hours (2, 3). This rejection process has membrane bound human complement regulatory proteins or reduced levels of Galα(1,3)Gal have been developed to overcome this type of rejection (4-6). In addition, immunoisolation of porcine cells by microencapsulation also appear to be promising in conferring protection against HAR (as shown in chapter IV, 7).

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[1] *A version of this chapter has been accepted for publication. Murray et al. 1999. Diabetes vol. 48.*

The subsequent immunological barrier to porcine-to-human xenotransplantation has not been well-defined. Cell-mediated cytotoxicity is one possible defense mechanism that could contribute to graft destruction. Human cell-mediated immune responses to porcine stimulator cells have been identified (8-10). It has also been shown that human CD8+ and CD4+ T cell subsets directly recognize swine SLA-I and SLA-II molecules, respectively *in vitro* (11-13). In addition, both NK and T cells in human peripheral blood lymphocyte populations can lyse pig lymphoid cells (14-16). These xenospecific cytotoxicity has been shown to be mediated through perforin/granzyme B and/or Fas/Fas ligand (FasL) pathways (17-19).

The susceptibility of NPI cells to lysis by human NK and cytotoxic T cells (CTL) is not known. Given the potential usefulness of NPI to treat human type 1 diabetes, in this study we examined the sensitivity of NPI cells to lysis by human peripheral blood lymphocytes (PBL), and reported that NPI cells are not susceptible to killing by either human NK or cytotoxic T cells *in vitro*.

V-B) MATERIALS AND METHODS

1. Preparation of NPI Cells

The method used to prepare NPI was previously described (1). Briefly, 1 to 3 day old Landrace-Yorkshire neonatal pigs (1.5 to 2.0 kg body weight) of either sex were anesthetized with Halothane and subjected to laparotomy and complete exsanguination. The pancreas was removed, minced, then digested with collagenase (2.5 mg/ml; Sigma, St Louis, MO). After filtration through a nylon screen (500 µm), the tissue was cultured for 7 to 9 days in HAM's F10 medium (Life Technologies, Burlington, ON) containing 10 mmol/l glucose, 50 µmol/l isobutylmethylxanthine (ICN Biochemicals, Montreal, PQ), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade, Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 µg/ml streptomycin. Freshly isolated pancreatic tissue and 7 to 9 day cultured NPI were dissociated into single cell suspensions by gentle agitation in calcium-free medium containing 15 µg/ml trypsin (Boehringer mannheim, Laval, PQ) and 4 µg/ml DNAse (Boehringer Mannheim). The cell suspension was filtered through a 63 µm nylon screen to remove cell clumps and then centrifuged through Percoll (Sigma) of density 1.040 g/ml to eliminate dead cells and debris. Single cell suspensions were subsequently used in the proliferation and CTL assays. To exclude an effect of trypsin used in the isolation of NPI cells on the NPI cells-lymphocyte interaction, splenocytes from neonatal pigs were treated with trypsin in parallel with the matched NPI cells and tested for susceptibility to lysis. These trypsin-treated splenocytes showed similar sensitivity as the untreated splenocytes to lysis.

2. Isolation of Human PBL

Blood was collected from human volunteers under a protocol approved by the University of Alberta research ethics committee using venopuncture or leukophoresis as described previously (20). Briefly, human mononuclear cells were isolated by centrifugation of blood diluted 1:2 with Hank's balanced salt solution (Life Technologies, Grand Island, NY) over Ficoll according to the instructions of the manufacturer (Nycomed Lymphoprep, Life Technologies). The mononuclear cells were resuspended in RPMI 1640 supplemented with either 10% pooled human AB or fetal bovine serum containing 100 U/ml penicillin and 100 μg/ml streptomycin then depleted of monocytes using a 1 hour incubation over fibronectin-coated plastic dishes at 37 °C to derive the PBL (20). In the proliferation experiments, the PBL were incubated over fibronectin-coated dishes a second time prior to use. In some experiments, CD4+ or CD8+ enriched lymphocyte populations were obtained from the PBL by negative selection using a cocktail of anti-human IgG and anti-CD16-conjugated immunomagnetic beads according to the manufacturer's instructions (Perspective Diagnostics, Cambridge, MA). To further deplete contaminating cell populations, the cell suspension was then incubated with a cocktail of anti-HLA class II and either OKT4 or OKT8 mAb as ascites at 4 °C for 30 minutes. The antibody-labeled cells were then washed and incubated with goat anti-mouse IgG-coated magnetic beads and removed by negative selection as directed by the manufacturer (Immunotech, Westbrook, ME). Enriched populations contained <1% of HLA-DR+ and CD4+ or CD8+ cells, respectively when anlyzed by flow cytometry. The preparations were functionally depleted of professional antigen-presenting cells (APC) since peak ^{3}H-methyl thymidine incorporation after stimulation with phytohemagglutinin (PHA; 0.5 µg/ml) was <10% maximal incorporation.

3. PBL Proliferation Assay

Single cell suspensions of NPI cells received 5,000 rad irradiation from a gamma cell irradiator (Nordion International, Kanata, ON), then 1x10^5 cells were aliquoted to each round bottom well (Falcon, Lincoln Park, NJ). Pilot studies indicated that this number of stimulator cells produced the optimal proliferation of PBL. Whole PBL or T cell subsets were then co-cultured with NPI cells for the indicated time at 1x10^5 cells/well for PBL and CD4+ T cells, or 2x10^5 cells/well for CD8+ T cells, in replicates of 4 for each group. Each well was pulsed with 1 µCi of ^{3}H-methyl thymidine and fluoro-deoxyuridine (1 µg/ml final concentration) for 24 hours, then lysed by a freeze/thaw cycle and harvested onto glass microfibre filters. Counts were determined using a liquid scintillation counter (Beckman, Fullerton, CA).

4. Cytotoxicity Assay

Cell-mediated cytotoxicity assay was performed as described previously (20). Briefly, NPI and Jurkat cells were suspended in media at 4x10^5 cells/ml, then labelled with ^{111}In oxine at a final specific activity of 40 µCi/ml for 1 hour at 37 °C. The cell suspension was washed once, then incubated in media for an additional hour and washed again before use. Neonatal porcine splenocytes were suspended at 3x10^6 cells/ml, then labeled with ^{111}In oxine at a final specific activity of 120 µCi/ml then handled like the NPI cells. NPI cells or Jurkat (2x10^4), or neonatal porcine splenocyte (5x10^4) target cells, were plated into U-bottom microwell, then effector PBL were added to achieve the desired effector to target cell ratio in a final volume of 200 μl/well. The cell suspensions were gently mixed, then centrifuged at 50g for 5 minutes. After incubation at 37°C for 4 to 6 hours as indicated, SDS was added to one replicate group at a final concentration of 0.1%. Eighty microlitres of supernatant was harvested from each well and the release of ^{111}In was determined using a LKB gamma counter (Wallac, Gaithersburg, MD). The percent specific release was calculated using the following formula:

$$\% \text{ specific release} = \frac{\text{experimental release - spontaneous release}}{\text{total release - spontaneous release}} \text{ X } 100$$

The data points shown represent the mean of quadruplicate wells. Spontaneous release was always less than 20%. The standard deviation of each data point shown was less than 10%.

Where indicated effector cells were pre-incubated with recombinant soluble FasR-Ig or isotype-matched control Ig for 30 minutes at 37°C before use. Soluble FasR-Ig was also used at 30 μg/ml during the co-culture of the effector PBL and target cells. In some experiments the BW cell line transfected to stably express human Fas-ligand at high density was used against Jurkat target cells to confirm the blocking activity of FasR-Ig. Perforin-mediated cytolysis was inhibited using media with 1 mmol/l $MgCl_2$ and 2 mmol/l EGTA to chelate calcium. In these assays the effector PBL were washed with this media three times, and the target cells were similarly washed twice with calcium-free media after they had been loaded with ^{111}In oxine label before use in the experiment. In other groups perforin-mediated cytolysis was blocked by pre-incubating the PBL effector cells in media containing 10 nmol/l concanamycin A at 37°C for 5 hours before use (21).

5. Indirect Immunofluorescence and Immunocytochemical Analyses

The expression of SLA-I and SLA-II antigen on the surface of NPI cells and neonatal porcine splenocytes was measured by indirect immunofluorescence and flow cytometry. Cells (10×10^6) were stained with either mouse anti-porcine SLA I mAb 74-11-10 (10 μg/ml; VMRD, Pullman, WA) or mouse anti-porcine SLA II mAb MSA-3 (10 μg/ml; VMRD) for 1 hour in PBS with 2% goat serum at 4 °C, then washed twice, and stained with FITC-conjugated sheep anti-mouse IgG. Mouse myeloma immunogloblin (Cappel, Durham, NC) was used as irrelevant mAb for negative control. Fluorescence intensity was quantified using a FACScan flow cytometer (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL) and fluorescence emitted by FITC-labeled cells was selected as the sorting parameter. Neonatal porcine splenocytes served as positive control. The efficacy of the cell separation was evaluated by re-analyzing each population by flow cytometry to detect the relative percentage of SLA-I-positive cells in each population.

The cellular composition of SLA-I positive sorted populations was determined by immunohistochemical staining for insulin, glucagon, somatostatin, and pancreatic polypeptide after adherence of the cells on adhesive-coated slides and fixed in Bouin's solution. The presence of pancreatic hormones in each cell population was detected using guinea pig anti-porcine insulin antibody (1:1,000 dilution; Dako Diagnostics, Mississauga, ON) followed by biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA), rabbit anti-porcine glucagon antibody (1:900 dilution; Dako Diagnostics); rabbit anti-human somatostatin antibody (1:1,000 dilution; Dako Diagnostics); rabbit anti-human pancreatic polypeptide antibody

(1:1,000 dilution; Dako Diagnostics) and biotinylated goat anti-rabbit IgG secondary antibody (1:200 dilution; Vector Laboratories). The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) method was used and developed with 3,3-diaminobenzedinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) to produce a brown color.

6. Statistical Analysis

The data were analyzed using SPSS 6.1 software (SPSS Inc., Chicago, IL) running on a Macintosh computer (Apple, Cupertino, CA). Significant differences in the mean between groups were tested using ANOVA at a p value of <0.05.

V-C) RESULTS

1. Recognition of NPI Cell Xenoantigen by Human Lymphocytes

To determine if human lymphocytes are able to recognize swine xenoantigen in the NPI cell preparation, human lymphocytes depleted of human APC were stimulated with irradiated NPI cells. Pilot experiments indicated that optimal proliferation of the human lymphocytes was obtained using 1×10^5 stimulator NPI cells/well. The magnitude and kinetic profile of the proliferative response against NPI cells was compared to the response against splenocytes isolated from the same donor pig (Figure 5-1). Human lymphocytes in four separate responder/donor pairs proliferated in response to the neonatal porcine splenocytes. The magnitude of the peak response to NPI stimulator cells ranged from approximately 50 to 100% of the peak proliferation detected using matched neonatal porcine splenocytes as stimulator cells. However, the human anti-NPI cells response rose more slowly than the response against porcine splenocytes.

To determine if both CD4+ and CD8+ T cell substes were able to recognize pig xenoantigen and proliferate, we prepared populations of human PBL enriched for CD4+ or CD8+ T cells. Human CD4+ T cells proliferated in response to NPI stimulator cells (Figure 5-2, $p<0.05$ *vs* unstimulated CD4+ PBL). This response was inhibited when anti-SLA-II mAb was added to the wells (data not shown). In contrast, CD8+ T cells showed weak ^{3}H-methyl thymidine incorporation which was not different from proliferation seen in the absence of irradiated porcine stimulator cells (Figure 5-2). On the other hand, CD8+ T cell proliferation was detected following co-culture with either matched neonatal porcine splenocytes or aortic endothelial stimulator cells. These experiments indicate that CD4+ subset accounts for most of the response seen using the whole PBL population.

2. Human NK and CTL-Mediated Lysis of NPI Cells

One mechanism by which xenografts are injured is through direct cell-mediated cytotoxicity induced by NK cells and CTL. When NPI cells or matched neonatal porcine splenocytes were exposed to freshly isolated human PBL, neonatal porcine splenocytes, but not NPI cells were lysed by NK cells (Figure 5-3; $p<0.05$). Similarly, when NPI cells or matched neonatal porcine splenocytes were exposed to human PBL that were co-cultured first with neonatal porcine splenocytes for 5 to 7 days to promote maturation of precursor CTL directed against swine xenoantigens, these CTL lysed porcine splenocytes, but not NPI cells (Figure 5-4; $p<0.05$).

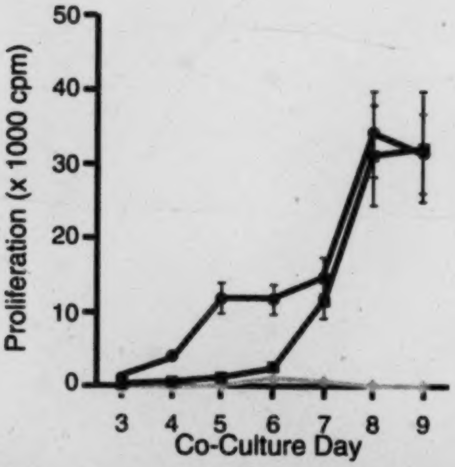

Figure 5-1. Proliferation of unstimulated human PBL (◆), or PBL stimulated by co-culture with neonatal porcine splenocytes (●), or NPI cells (■). Co-cultures were prepared as described in Methods, and labeled with 1 µCi ^{3}H-methyl thymidine for 24 hours prior to harvest. Data shown are representative of 4 experiments using different responder/porcine stimulator combinations.

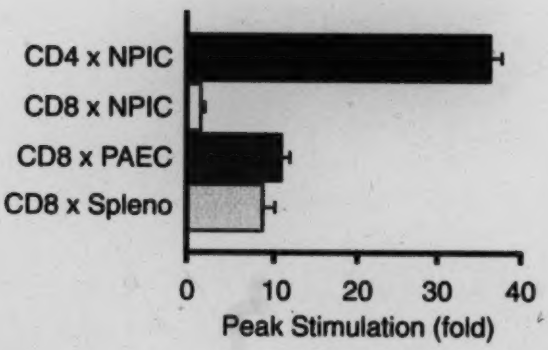

Figure 5-2. Human CD4+ and CD8+ T cell proliferation in response to NPI cell stimulation. CD4+ and CD8+ T cells were isolated by negative selection and co-cultured with NPI cells as detailed in Methods. The co-cultures were labeled with 1 µCi ^{3}H methyl thymidine for 24 hours prior to harvest. The stimulation indices shown are compiled from 2 separate experiments using either CD4+ or CD8+ responder lymphocytes.

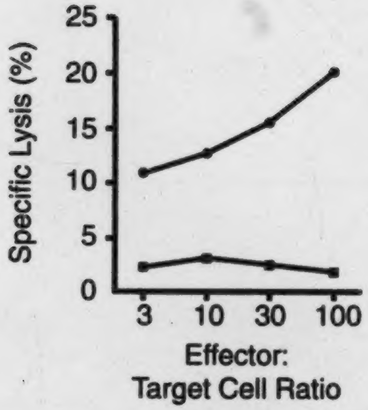

Figure 5-3. Human NK cell activity against NPI cells or neonatal porcine splenocytes. Freshly isolated PBMC were incubated with ^{111}In oxine-labeled NPI cells (■) or matched neonatal porcine splenocytes (●) for 4 hours in the presence of 10% human AB serum. Specific release of ^{111}In was determined as described in Methods. The data are representative of 2 separate experiments.

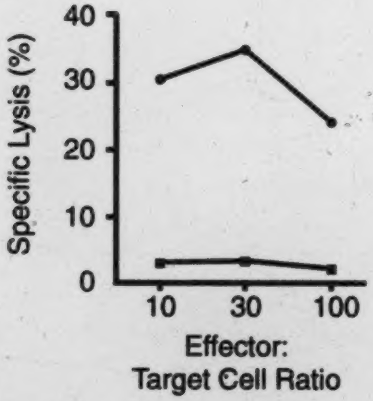

Figure 5-4. Human CTL lyse neonatal porcine splenocytes, but not NPI cells. Human PBL were cultured with neonatal porcine splenocytes for 7 days, then tested for the ability to lyse neonatal porcine splenocytes (●) or NPI cells (■) from the same donor pig as determined by ^{111}In release assay described in Methods.

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3. Expression of SLA-I and SLA-II on NPI Cells

Since previous work has described human T cell proliferation to both SLA-I and SLA-II molecules, we determined the MHC expression on NPI cells. Using flow cytometry, we found 35.9±6.5% (Table 5-1) of NPI cells express SLA-I antigen, but very few cells express SLA-II (0.4±0.4%). Treatment of NPI cells with supernatant of porcine splenocytes stimulated with PHA (Figure 5-5), enhanced both SLA-I (81.8±5.5%) and SLA-II expression (25.1±11.4%).

The cellular composition of SLA-I-positive and -negative cells was determined by immunocytochemical staining. Significantly more insulin-positive cells (60.0±5.2%) are found in SLA-I-positive population than in SLA-I-negative cells (22.8±5.6; Table 5-2). There was no significant difference in the number of cells that are positive for other pancreatic hormones between the two groups.

Table 5-1. Expression of SLA-I and SLA-II on NPI cells and splenocytes.

	Percentage of total cells	
	SLA-I	SLA-II
Control	35.9±6.4 (5)	0.4±0.4 (3)
Supernatant-treated	81.1±5.5 (5)*	25.1±11.4 (3)*
Splenocytes	86.0±8.3 (5)*	35.3±10.1 (3)*

Data are means±SEM of (n) independent experiments. NPI cells and neonatal porcine splenocytes were stained with FITC-conjugated mouse anti-porcine SLA-I or SLA-II mAbs and analysed by flow cytometry as described in Methods. Control represents NPI cells not treated with PHA supernatant. Significant differences between groups were analyzed using ANOVA. *p<0.01 *vs* control.

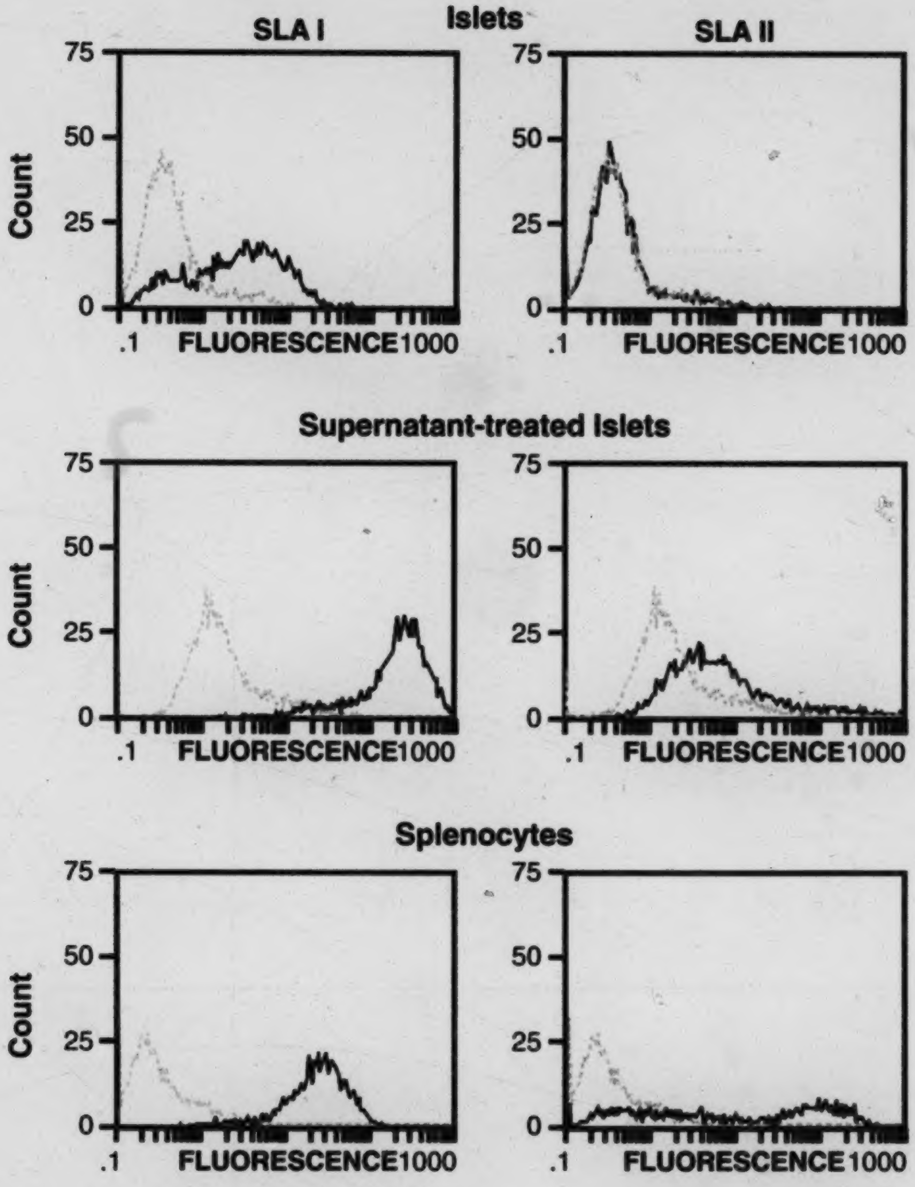

Figure 5-5. SLA-I and SLA-II expression on NPI cells. NPI cells were stained with mAb to either SLA-I or SLA-II (heavy line). Irrelevant mAb (dash line) was used as negative control.

Table 5-2. Cellular composition of SLA I-positive or -negative NPI cells.

Islets	n	Percentage of total cells Insulin	Glucagon	Pancreatic Polypeptide	Somatostatin
Unsorted	4	44.2±3.0	28.0±2.8	7.3±1.8	8.3±1.0
SLA I-positive	5	60.0±5.2*	17.3±2.6	6.5±2.2	5.7±1.5
SLA I-negative	4	22.8±5.6	27.4±10.2	10.5±3.7	7.6±2.3

Data are means±SEM of n independent experiments. NPI cells were fixed in Bouin's solution and immunostained with appropriate antibodies as described in Methods. Statistical differences between groups were calculated using ANOVA; *$p<0.01$ *vs* unsorted and SLA I-negative NPI cells.

4. The Effect of Pro-Inflammatory Cytokine Pre-Treatment of NPI Cells on Anti-NPI Cells Activity

Since low MHC or adhesion molecule density on the NPI target cells might impair the ability of cytolytic lymphocytes to deliver a lethal hit, we determined the effect of pre-treatment of NPI cells with porcine cytokine-rich supernatant (which increases MHC expression on NPI cells) on the susceptibility of NPI cells to lysis by human CTL. Pre-treatment of NPI cells with cytokine-rich supernatant failed to render the NPI cells more sensitive to lysis by human CTL (Figure 5-6). Similar findings were obtained when NPI cells were pre-treated with PHA-stimulated human splenocyte supernatant. Lysis of NPI cells was elicited when human PBL were stimulated with PHA for 24 hours prior to exposure to NPI cells (Figure 5-7). NPI cells ^{111}In release was <5% following incubation with either resting (unstimulated) or splenocyte-sensitized PBL, but was significantly increased when PHA was added.

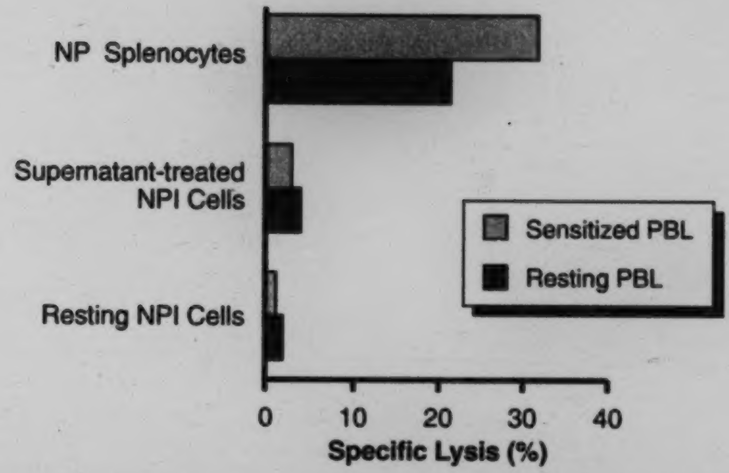

Figure 5-6. Cell-mediated lysis of NPI cells after treatment with cytokine-rich supernatanat. Human PBL were cultured with neonatal porcine splenocytes for 7 days, then tested for the ability to lyse isogeneic neonatal porcine splenocytes, NPI cells, or NPI target cells which were pre-treated with stimulated porcine splenocyte supernatant as a source of porcine cytokines. An effector:target cell ratio of 30:1 was used in this experiment. The data are representative of two experiments.

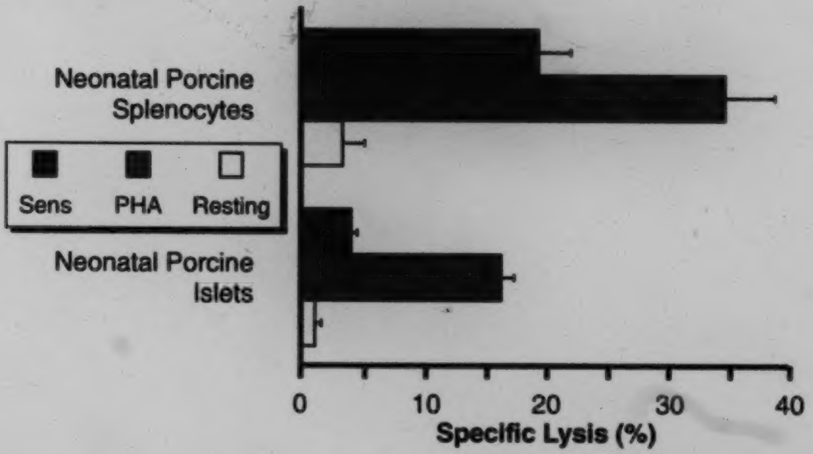

Figure 5-7. Lysis of NPI cells and neonatal porcine splenocytes is enhanced by co-culture in the presence of PHA. Human PBL were cultured with porcine splenocytes for 5 days, then tested for the ability to lyse porcine target cells. Target NPI cells and splenocytes were isolated from the same animal, then cultured for 7 days before use in the cytolysis assay. Stimulated human PBL were incubated with the ^{111}In-labeled target cells in the absence or presence of PHA. The data are representative of 3 experiments.

5. Determination of the Effector Mechanism used by CTL to Lyse Neonatal Porcine Splenocytes

Cytotoxic lymphocytes can use several effector molecules to kill the target cell, including perforin and granzyme, Fas/Fas-ligand, and TNF. Fas-and TNF-mediated killing are effective against some target cells, such as cells of bone marrow origin, but may not act to kill NPI cells. This death mechanism has been shown to Ca^{2+}-independent (21, 22). On the other hand, the perforin and granzyme mechanism is a more general tool used by CTL to induce death of any target cell. This death mechanism is dependent on Ca^{2+} (23). We determined the mechanism used by CTL to kill neonatal porcine splenocytes. Lysis of neonatal porcine splenocytes was blocked completely by either pre-treatment of the PBL with the V-ATPase inhibitor concanamycin A (24) or chelation of free calcium in the co-culture medium during the effector phase of the assay (Figure 5-8). In contrast, the blocking Fas receptor chimeric protein (FasR-Ig) inhibited human PBL lysis of neonatal porcine splenocytes by less than 10%, but completely inhibited Fas-mediated lysis of Jurkat cells. These data indicate that human PBL use perforin and granzymes to lyse neonatal porcine splenocytes.

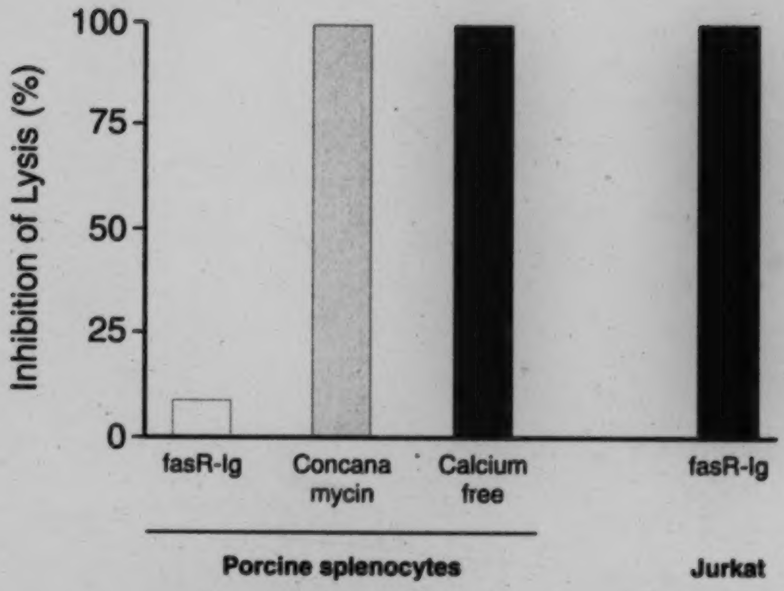

Figure 5-8. Lysis of neonatal porcine splenocytes was inhibited completely by either pre-treatment of the PBL with the V-ATPase inhibitor concanamycin A or chelation of free calcium. Fas receptor chimeric protein (FasR-Ig) inhibited lysis of neonatal porcine splenocytes by <10%, but completely inhibited Fas-mediated lysis of Jurkat cells by BW cell line which stably expressed human Fas ligand.

V-D) DISCUSSION

We found that purified human CD4+ PBL proliferate in response to işlet cell xenoantigens, and that blocking mAb to porcine MHC class II molecules attenuates the response. In agreement with Heiser *et al.* (25), we found a small subpopulation of SLA-II-positive cells among the NPI cells stimulator cell (Table 5-1). Previous work has shown that human CD4+ T cells are capable of directly recognizing SLA class II molecules, and that the frequency of such xenoreactive T cells exceeds the frequency of alloreactive T cells in the human PBL compartment (20, 26, 27). Taken together, these data suggest that human CD4+ T cells are stimulated by SLA class II-positive cells in the NPI cells.

We report the unexpected observation that neither freshly isolated human PBL with xenoreactive NK cell activity nor PBL sensitized with neonatal porcine splenocytes were able to kill NPI cells, but did lyse isogeneic neonatal porcine splenocytes. Previous observations in rodent models of both autoimmune diabetes and allogeneic islet transplantation have demonstrated that islet cells are susceptible to cell-mediated cytotoxicity *in vitro* and injury *in vivo* (28-34). Indeed recent experiments have shown that diabetes-prone mice defective in perforin gene expression have a markedly reduced incidence of hyperglycemia, suggesting that cell-mediated cytotoxic injury of the β cell is a critical event in the development of diabetes (35, 36).

Earlier work has shown that both NK and CD3+ lymphocytes present in the human PBL compartment are capable of inducing lysis of porcine lymphoid cell targets (14-16). The NK cell component was found to contribute most of this activity and to function without prior activation. Nevertheless, studies of the interaction of human NK cells with nonlymphoid porcine target cells, such as endothelial cells, have shown either no lysis or lysis only at high effector to target cell ratios (12, 37).

Cytolytic T lymphocytes have also been found to function against porcine target cells. Lucas *et al.* (38) found human CTL-mediated lysis of porcine PHA-stimulated PBMC targets was inhibited to only a minor degree by blocking anti-CD4 antibody suggesting that CD8+ T cells accounted for most of the lytic activity (27). Similarly, other groups have reported SLA-I restricted cytolysis of porcine endothelial (39) or lymphoid target cells (15) by human lymphocytes.

The explanation for the inability of either NK or CTL to effect lysis of NPI cells is not clear. NK cells receive both stimulatory and inhibitory signals from the target cell through a series of receptors (40). For example, recognition of the target cell through lectin-binding domains of the NKR-P1 receptor family is required for NK cell cytolytic function (40, 41). Tissue-specific differences in the interaction of these stimulatory or inhibitory receptors for ligands on xenogeneic porcine target cells, perhaps as a result of different post-transcriptional modifications of a receptor protein, might account for the different susceptibility to NK cell-mediated lysis of the porcine splenocytes and NPI cells. Differential tissue expression of these ligands, however, has not been reported.

We were not able to detect either significant CD8+ T cell proliferative responses to NPI cell xenoantigen or CTL-mediated injury of the NPI cells to provide direct evidence for CD8+ T cell recognition of porcine MHC class I expressed by the NPI cells. Since SLA class I-restricted proliferation of purified populations of human CD8+ T cells to porcine professional antigen presenting cells has been observed previously, accessory molecules required for efficient IL-2 production by the CD8+ lymphocytes may be lacking on the NPI cells (20, 26, 27). SLA-I expression on NPI cells was relatively low, nevertheless, few MHC class I molecules are required for target cell recognition by CD8+ T cells, hence low expression of SLA-I is unlikely to explain the lack of activation or killing. Moreover, SLA-I expression was further induced on a subpopulation of NPI cells after cytokine treatment, but did not enhance cytolytic killing by sensitized CTL. Although critical residues in the binding region of CD8 are altered in the α3 domain of the pig MHC class I molecule compared to the human homolog (42), this has not precluded CD8+ T cell lysis of porcine splenocyte or endothelial target cells (38). Finally, saturation of the SLA-I molecules with islet-specific peptides is unlikely to explain the lack of NPI cell lysis, since both NK and cytotoxic T lymphocytes fail to lyse the NPI cell targets.

One explanation for the failure of NK cells and CD8+ T cells to kill NPI cells may be differences in adhesion molecule expression between the splenocyte and NPI cells. The efficiency of both NK cell- and CTL-mediated lysis is known to be enhanced by interaction with adhesion receptors expressed by the target cell (43). Expression of ligands for human LFA-1, CD2, and CD28 have been reported on porcine APC (20, 27). However, little is known regarding the expression of these molecules on other pig tissues, or the expression of other adhesion molecules in the pig. A critical molecule used by NK cells and CTL to adhere to the target cell is CD54, a ligand for LFA-1. Both rodent and human islet cells constitutively express CD54, and upregulation of CD54 expression following stimulation with proinflammatory cytokines renders these islet cells more susceptible to lysis *in vitro* (44). Expression of pig CD54 on NPI cells could not be tested directly since reagents are not available. However, neither pre-treatment of NPI cells with recombinant human TNF nor the pig cytokine-rich splenocyte supernatant enhanced NPI cells killing.

We considered the possibility that resistance of NPI cells to human cytotoxic lymphocytes may be dependent on the duration of the co-culture used in the lysis assay. However, we found that increasing the time of co-culture of the sensitized PBL and NPI cells to 6 hours to increase the sensitivity of the assay still resulted to no lysis of NPI cells (data not shown). Incubation of target cells longer than 6 hours resulted to non-specific release of the label from the target cells and limited longer assay conditions.

Taken together, the data presented here demonstrate NPI cells are not susceptible to lysis induced by human PBL as measured by ^{111}I release assay. It is not clear if other cellular porcine grafts show similar resistance to cytolytic injury. Our results confirm earlier reports of CD4+ T cell recognition of the porcine xenografts and suggest that a CD4+ T cell-directed DTH response stimulated through either the direct or the indirect antigen presentation pathway may represent a more significant barrier to the transplantation of NPI cells into human recipients.

V-E) REFERENCES

1. Korbutt GS, Elliott JF, Ao Z, *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-21129.

2. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC. Anti-pig IgM antibodies in human serum react predominantly with Gal (α 1-3) Gal epitopes. Proc Natl Acad Sci USA 1993; 90: 11391-11395.

3. Cooper DKC, Good AH, Koren E, *et al.* Identification of α-galactosyl residues and other carbohydrate epitopes that are bound by human anti-pig antibodies: relevance to discordant xenografting in man. Transplant Immunol 1993; 1: 198-205.

4. Rosengard AM, Cary N, Horsley J, *et al.* Endothelial expression of human decay accelerating factor in transgenic pig tissue: a potential approach for human complement inactivation in discordant xenografts. Transplant Proc 1995; 27: 326.

5. Sandrin MS, Fodor WL, Mouhtouris E, *et al.* Enzymatic remodeling of the carbohydrate surface of a xenogeneic cell substantially reduces human antibody binding and complement-mediated cytolysis. Nat Med 1995; 12: 1261.

6. Sharma A, Okabe J, Birch P, *et al.* Reduction in the level of Gal (α 1,3)Gal in transgenic mice and pigs by the expression of an α (1,2)fucosyltransferase. Proc Natl Acad Sci USA 1996; 93: 7190.

7. Rayat GR, Rajotte RV, Ao Z, Flashner M, Korbutt GS. Microencapsulation of neonatal porcine islets: long-term reversal of diabetes in nude mice and *in vitro* protection from human antibody/complement-mediated cytolysis. (Submitted to Tansplantation)

8. Yamada K, Seebach JD, DerSimonian H, Sachs DH. Human anti-pig T-cell mediated cytotoxicity. Xenotransplantation 1996; 3: 179-187.

9. Sawada T, DellaPelle PA, Seebach JD, *et al.* Human cell-mediated rejection of porcine xenografts in an immunodefficient mouse model. Transplantation 1997; 63: 1331-1338.

10. Yi S, Feng X, Wang Y, Kay TWH, *et al.* CD4+ cells play a major role in xenogeneic human anti-pig cytotoxicity through the Fas/Fas ligand lytic pathway. Transplantation 1999; 67: 435-443.

11. Inverardi L, Samaja M, Motterlini R, *et al.* Early recognition of a discordant xenogeneic organ by human circulating lymphocytes. J Immunol 1992; 149: 1416.

12. Malygiune AM, Saadi S, Platt JL, Dawson JR. Human natural killer cells induce morphologic changes in porcine endothelial cell monolayers. Transplantation 1996; 61: 161-164.

13. Watier H, Guillaumin JM, Piller F, *et al.* Removal of terminal α-galactosyl residues from xenogeneic porcine endothelial cells. Decrease in complement-mediated cytotoxicity but persistence of IgG1-mediated antibody-dependent cell-mediated cytotoxicity. Transplantation 1996; 62: 105-113.

14. Kirk AD, Li RA, Kinch MS, *et al.* The human antiporcine cellular repertoire: *In vitro* studies of acquired and innate cellular responsiveness. Transplantation 1993; 55: 924-931.

15. Yamada K, DerSimonian H, Sachs DH. The mechanism of xenogeneic cell-mediated lympholysis between human and pig cells. Transplant Proc 1996; 28: 757.

16. Donnelly CE, Yatko C, Johnson EW, Edge AS. Human natural killer cells account for non-MHC class I-restricted cytolysis of porcine cells. Cell Immunol 1997; 175: 171-178.

17. Wecker H, Winn HJ, Auchincloss H Jr. CD4+ T cells, without CD8+ or B lymphocytes, can reject xenogeneic skin grafts. Xenotransplantation 1994; 1: 8-16.

18. Smyth MJ, Thia KYT, Kershaw MH. Xenogeneic mouse anti-human NK cytotoxicity is mediated via perforin. Xenotransplantation 1997; 4: 78-84.

19. Smyth MJ, Kershaw MH, Trapani JA. Xenospecific cytotoxic T lymphocytes: potent lysis *in vitro* and *in vivo*. Transplantation 1997; 63: 1171-1178.

20. Murray AG, Khodadoust MM, Pober JS, Bothwell AL. Porcine aortic endothelial cells activate human T cells: Direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD28. Immunity 1994; 1: 57-63.

21. Trenn G, Takayama H, Sitkovsky MV. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. Nature 1987; 330: 72-74.

22. Ostergaard HL, Kane KP, Mescher MF, Clark WR. Cytotoxic T lymphocytes mediated lysis without release of serine esterase. Nature 1987: 71-72.

23. Yamada K, Takane-Gyotoku N, Yuan X *et al.* Mouse islet cell lysis mediated by interleukin-1-induced Fas. Diabetologia 1996; 39: 1306-1312.

24. Kataoka T, Takaku K, Magae J, *et al.* Acidification is essential for maintaining the structure and function of lytic granules in CTL. J Immunol 1994; 153: 3938-3947.

25. Heiser A, Ulrichs K, Eckstein V, Muller-Ruchhltz W. Xenogeneic cellular response of human lymphocytes to porcine lymphocytes and isolated porcine pancreatic islets. Transplant Proc 1992; 24: 2881-2882.

26. Bravery CA, Batten P, Yacoub MH, Rose ML. Direct recognition of SLA- and HLA-like class II antigens on porcine endothelium by human T cells results in T cell activation and release of interleukin-2. Transplantation 1995; 1024-1033.

27. Rollins SA, Kennedy SP, Chodera AJ, *et al.* Evidence that activation of human T cells by porcine endothelium involves direct recognition of porcine SLA and costimulation by porcine ligands for LFA-1 and CD2. Transplantation 1994; 57: 1709-1716.

28. Knospe S, Kohler E, Kloting I. Cell-mediated immune reactions against islets of Langerhans in diabetes-prone BB rats. Exp Clin Endocrinol 1987; 89: 290-296.

29. Stock PG, Meloche M, Ascher NL, *et al.* Generation of allospecific cytolytic T-lymphocytes stimulated by pure pancreatic beta-cells in absence of Ia+ dendritic cells. Diabetes 1989; 38: 161-164.

30. Nagata M, Yokono K, Hayakawa M, *et al.* Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice. J Immunol 1989; 143: 1155-1162.

31. Nakamura N, Woda BA, Tafuri A, *et al.* Ortaldo J, Chick W, Handler ES, Mordes JP, Rossini AA. Intrinsic cytotoxicity of natural killer cells to pancreatic islets *in vitro*. Diabetes 1990; 39: 836-843.

32. Hayakawa M, Yokono K, Nagata M, *et al.* Morphological analysis of selective destruction of pancreatic beta-cells by cytotoxic T lymphocytes in NOD mice. Diabetes 1991; 40: 1210-1217.

33. Nagata M, Santamaria P, Kawamura T, *et al.* Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta-cells in nonobese diabetic mice. J Immunol 1994; 152: 2042-2050.

34. Yoneda R, Yokono K, Nagata M, *et al.* CD8 cytotoxic T-cell clone rapidly transfers autoimmune diabetes in very young NOD and MHC class I-compatible scid mice. Diabetologia 1997; 40: 1044-1052.

35. Kagi D, Odermatt B, Ohashi PS, *et al.* Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. J Exp Med 1996; 183: 2143-2152.

36. Kagi D, Odermatt B, Seiler P, *et al.* Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. J Exp Med 1997; 186: 989-997.

37. Goodman DJ, Von Albertini M, Willson A, *et al.* Direct activation of porcine endothelial cells by human natural killer cells. Transplantation 1996; 61: 763-771.

38. Lucas PJ, Shearer GM, Neudorf S, Gress RE. The human antimurine xenogeneic cytotoxic response. I. Dependence on responder antigen presenting cells. J Immunol 1990; 144: 4548-4554.

39. Shishido S, Naziruddin B, Howard T, Mohanakumar T. Recognition of porcine majorhistocompatibility complex antigens by human CD8+ cytolytic T cell clones. Transplantation 1997; 64: 340-346.

40. Lanier LL. Natural killer cells: from no receptors to too many. Immunity 1997; 6: 371-378.

41. Inverardi L, Clissi B, Stolzer AL, *et al.* Human natural killer lymphocytes directly recognize evolutionary conserved oligosaccharide ligands expressed by xenogeneic tissues. Transplantation 1997; 1318-1330.

42. Sullivan JA, Oettinger HF, Sachs DH, Edge ASB. Analysis of polymorphism in porcine MHC class I genes. J Immunol 1997; 159: 2318-2326.

43. Storkus WJ, Dawson JR. Target structures involved in natural killing (NK): Characteristics, distribution, and candidate molecules. Crit Rev Immunol 1991; 10: 393-416.

44. Yagi N, Yokono K, Amano K, *et al.* Expression of intercellular adhesion molecule 1 on pancreatic beta-cells accelerates beta-cell destruction by cytotoxic T-cells in murine autoimmune diabetes. Diabetes 1995; 44: 744-752.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSION

VI-A) GENERAL DISCUSSION

Insulin therapy and dietary regulation have improved the quality of life and extended life expectancy of patients with type 1 diabetes, but neither cure this disease nor prevent its complications unless intensive insulin therapy is used. The replacement of pancreatic endocrine tissue by islet transplantation offers a physiological means for precise restoration of euglycemia in patients with type 1 diabetes. Over the past 16 years, significant advances have been made in the number and purity of islets that can be harvested from the human pancreas (1-6). Reports of both short-term and long-term insulin-independence, following human islet allotransplantation (7-13) has encouraged several centers in the world (including the University of Alberta) to continue clinical trials of islet transplantation in patients with type 1 diabetes. However, if islet transplantation is to become a widespread treatment for these patients, the supply of donor organs will become a major limitation. In order to solve this problem, the use of other source of insulin-producing tissue is being considered. Among them are porcine (14-19) and bovine (20) islets, fish Brockman bodies (21), genetically-engineered insulin-secreting cell lines (22-24), and *in vitro* production of human fetal (25) and adult β cells (26). Pig pancreas is a practical source of abundant islets because pigs are inexpensive, readily available, they exhibit morphological and physiological characteristics similar to humans. Pig insulin is also structurally similar to human insulin with a difference in one amino acid. Pig insulin has also been used safely for treating patients with type 1 diabetes for many years.

Isolation of islets from adult (27-34) and fetal (15, 16) pigs have been reported. However, in the case of adult pig islets, these tissues are fragile and difficult to maintain in tissue culture. In addition, several factors such as breed and diet of the animals greatly affect the final yield of islets (28, 31). In contrast, tissue culture of collagenase digested fetal porcine pancreas produces viable islet-like cell clusters, but it took more time (two months post-transplantation) to reverse hyperglycemia when transplanted into diabetic nude mice. The development of a reliable method for isolating islets from neonatal pigs by Korbutt *et al.* (19) has offered another possible source of insulin-producing tissue for transplantation into diabetic patients. Unlike adult pig islets, neonatal porcine islets (NPI) are robust and can be easily maintained in tissue culture. In addition, these islets were shown to exhibit growth potential and secrete significant amounts of insulin in response to *in vitro* glucose challenge. When transplanted into diabetic nude mice, hyperglycemia was reversed between 6-8 weeks post-transplantation. Taken together, these characteristics make NPI an attractive source of insulin-producing tissue for clinical transplantation.

As we contemplate on using NPI in the future, there are several factors that we need to address. In particular, the immunological responses to NPI must be examined. This thesis involved several studies which focused on characterization of NPI as an alternative source of insulin-producing tissue for transplantation into patients with type 1 diabetes. First, the humoral arm of the human immune response was examined. Like pig solid organs, NPI also express the xenoantigen Galα(1,3)Gal and they are susceptible to damage induced by human antibody and complement *in vitro*. Studies presented in Chapter II that alternative complement pathway plays a major role in killing of NPI cells. This finding is in agreement with other studies (35-37) where destruction of pig cells occur even in the absence of xenoreactive antibodies.

The expression of Galα(1,3)Gal on porcine islet cells has been controversial. The studies presented in Chapter III have shown for the first time that Galα(1,3)Gal expression on porcine islet endocrine cells is age-dependent. This may explain in part the discrepancies between the studies presented in Chapter II (32) and other studies (38, 39) which used adult pig islets. It was also speculated in Chapter III that Galα(1,3)Gal may play a role during the differentiation and maturation of islet cells into insulin-producing cells. This role may include cell-to-cell communication so islet cells are arranged in certain orientation (*i.e.* β cells in the center surrounded by α, δ, and PP-secreting cells within the islet) and once the islet is formed Galα(1,3)Gal may no longer be needed. In Chapter IV, microencapsulation was shown to protect NPI from damage induced by human preformed natural antibodies and complement. Studies presented in this chapter also showed that microencapsulated NPI can reverse the hyperglycemic state of diabetic nude mice.

The other immunological hurdle which we investigated is the cell-mediated immune response to NPI. From the results obtained in this Chapter V it was predicted that direct cell-mediated injury of transplanted NPI by either human NK cells or CTL, may not be an important mechanism of islet xenograft rejection in the neonatal pig-to-human combination. Purified human CD4+, but not CD8+ PBL proliferate in response to NPI cell antigens, and that blocking mAb to porcine MHC class II molecules reduces the response. This suggests that human CD4+ T cells are stimulated by SLA class II-positive cells in the NPI preparation. It was also demonstrated in Chapter V that neither freshly isolated human PBL with xenoreactive NK cells nor PBL sensitized with neonatal porcine splenocytes were able to kill NPI although the same PBL did lyse isogeneic neonatal porcine splenocytes. Previous observations in rodent models of both autoimmune diabetes and allogeneic islet transplantation have demonstrated that islet cells are susceptible to cell-mediated cytotoxicity *in vitro* and injury *in vivo* (40-46). The explanation for the inability of either NK cells or CTL to lyse NPI cells is not clear. One explanation for the failure of NK cells and CD8+ T cells to kill NPI cells may be differences in adhesion or co-stimulator molecule expression between the splenocytes and NPI cells.

In allograft rejection, both direct and indirect pathways are believed to play a role, although the direct pathway is thought to be most crucial at first, whereas, during xenograft rejection the indirect pathway is thought to be dominant, if not the exclusive role in activating host T cells (45). Gill *et al.* (47) have shown that elimination of donor APC from mouse islet allografts led to indefinite graft survival, while similar treatment of concordant rat islet xenografts had little benefit for survival. Furthermore, rejection of the islet xenografts showed a specific dependence on CD4+ T cells, and not to the presence of donor APC and host CD8+ T cells (47). In addition, adoptive transfer studies into immune-deficient C.B-17 SCID mice demonstrate that CD4+ T cells are both necessary and sufficient to reconstitute these animals for islet xenograft immunity (48). These studies suggest that rejection of xenografts occurs predominantly through the indirect pathway. This form of xeno-presentation would be expected to activate host CD4+ T cells specific for xenogeneic antigens in the context of self class II MHC. The indirect pathway would tend to result in a non-antigen-specific effector mechanism, possibly through the toxic effects of cytokines or other inflammatory mediators resulting from the encounter of CD4+ T cells with antigens presented by APC.

Whether CD4+ T cells play a major role in the destruction of NPI cells remains to be elucidated. *In vivo* studies designed to determine the susceptibility of NPI to both humoral and cell-mediated rejection must be tackled next. Moreover, the protection of NPI provided by microencapsulation against cell-mediated immune response must also be investigated to provide information on the possibility of using this strategy in preventing immune attack of NPI if transplanted into patients with type 1 diabetes.

The discussion about xenotransplantation seems incomplete without xenosis. The risk of infectious disease transmission from graft to recipient, and conceivably on to the new host population, remains a topic of lively debate but relatively little research. The risks can be reduced or eliminated by using specific-pathogen-free animal colonies but this approach will not work for the porcine endogenous retrovirus (PERV) because the genome of these viruses is in the germline of every pig (49). Multiple copies of PERV are integrated in the pig genome which suggests that breeding "clean" pigs will be extremely difficult (50). PERV particles are released spontaneously by cell lines originating from pig kidney, lymph node, testis, and fallopian tube (51-53). PERV have approximately 60% sequence homology to the gibbon ape leukemia and murine leukemia C-type retrovirus (54-58). Retroviruses result in lifelong infection (57) and reports that PERV from cell lines and porcine lymphocytes can infect human cells *in vitro*, (52, 54) have prompted the US Food and Drug Administration to put porcine xenograft trials on hold until previously exposed patients are assessed for PERV infection and until prospective monitoring of xenograft recipients is established. More recent studies (50, 58) in xenotransplantation had addressed these issues and promising results have been reported. Having established that the nested PCRs could detect single molecules of target sequence, Patience *et al.* (58) had analyzed DNA isolated from patients' peripheral blood mononuclear cells and found no evidence of pig or PERV DNA in two renal dialysis patients whose circulation had been linked extracorporeally to pig kidneys. In addition, they found no seroconversion for PERV-specific antibodies. Similarly, Heneine *et al.* (50) were unable to detect markers of PERV infection in 10 diabetic patients who had received porcine fetal islets despite the evidence for extended exposure to pig cells and despite concomitant immunosuppressive therapy. These studies are important because they suggest the absence of PERV infection in patients which received pig tissue as well as established useful methods for detection of possible PERV infection in patients given pig xenografts.

In summary, studies presented in this manuscript demonstrate that there are cells in the NPI that express Galα(1,3)Gal and this antigen is also present on endocrine cells which is contrary to what other studies have shown. We also showed that NPI cells are susceptible to lysis mediated by human complement and this can be avoided when NPI are contained in alginate microcapsule. We showed that expression of Galα(1,3)Gal porcine islet cells is age-dependent which may explain in part why other studies did not detect this antigen on endocrine cells. Finally, NPI cells were shown to be susceptible to lysis mediated by human PBL. Whether they are also susceptible to killing mediated by other type(s) of human immune cells is not known. These findings provide information on the feasibility of using NPI for transplantation into patients with type 1 diabetes, however, similar studies must also be performed in animal models.

VI-B) CONCLUSION

The finding that islet grafts can achieve and maintain long-term glucose homeostasis in human subjects has proven that islet transplantation can be a therapeutic option for management of type 1 diabetes. However, the shortage of human organ donors and the use of immunosuppressive drugs to prevent rejection of islet grafts, are one of the limiting factors for the application of islet transplantation in patients with this disease. The potential of neonatal pigs as a source of unlimited supply of insulin-producing tissue for clinical transplantation is promising and protocols on how to avoid the need for continuous use of immunosuppression in patients with type 1 diabetes must be thoroughly examined. In this manuscript we demonstrated that microencapsulation protects NPI from killing mediated by human complement. This is a promising finding, however, the efficacy of this strategy must still be tested in cell-mediated immunity and animal model. The issue of autoimmunity regarding porcine tissues has not been elucidated in the studies presented here, however, it is a very important issue that we also need to address. Why Galα(1,3)Gal disappears as NPI cells develop into insulin-producing β cells is a very intriguing question. Whether this antigen really plays a role during development of NPI cells remains to be determined. In this manuscript we transplanted NPI in normal Scid mice, we do not know whether Galα(1,3)Gal will also disappear when NPI are transplanted into diabetic animal. It would be very interesting to find out if islet cells from chemically-induced diabetic Scid mice also express Galα(1,3)Gal and what happens to this antigen when NPI mature in a diabetic animal. It would also be interesting to find out if Galα(1,3)Gal-knockout animal would have similar cell arrangement as that found in normal animal (non-knockout). These studies will give us information on the possible role of Galα(1,3)Gal in the development of islet cells. The safety and regulatory issues encompassing the use of animal tissue for clinical transplantation is a growing area of concern and whether any of the identified PERVs would pose a hazard for humans is not known. Research intended to define and quantify infectious risks must be further developed so progress in the development of xenotransplantation can be achieved with enhanced safety.

VI-C) REFERENCES

1. Lacy PE. Islet cell transplantation for insulin-dependent diabetes. Hosp Pract 1995; 30: 41-45.

2. Brandhorst D, Brandhorst H, Hering BJ, *et al.* Islet isolation from the pancreas of large mammals and humans: 10 years of experience. Exp Clin Endocrinol 1995; 103: 3-14.

3. Brandhorst H, Brandhorst D, Hering BJ, *et al.* Body mass index of pancreatic donors: A decisive factor for human islet isolation. Exp Clin Endocrinol 1995; 103: 23-26.

4. Bretzel RG, Hering BJ, Federlin KF. Islet cell transplantation in diabetes mellitus - from bench to bedside. Exp Clin Endocrinol 1995; 103: 143-159.

5. Calafiore R. Perspective in pancreatic and islet cell transplantation for the therapy of IDDM. Diabetes Care 1997; 20: 889-896.

6. Slover RH, Eisenbarth GS. Prevention of type 1 diabetes and recurrence of β-cell destruction of transplanted islets. Endocr Rev 1997; 18: 241-258.

7. Scharp DW, Lacy PE, Santiago JV, *et al.* Insulin independence after islet transplantation into type 1 diabetic patient. Diabetes 1990; 39: 515-518.

8. Warnock GL, Knetemen NM, Rayan E, *et al.* Normoglycemia after transplantation of freshly isolated and cryopreserved pancreatic islets in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1991; 34: 55-58.

9. Socci C, Falqui L, Davalli AM, *et al.* Fresh human islet transplantation to replace pancreatic endocrine function in type 1 diabetic patients: Report of six cases. Acta Diabetol 1991; 128: 151-157.

10. Warnock GL, Kneteman NM, Rayan EA, *et al.* Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 89-95.

11. Ricordi C, Tzakis AG, Carroll PB, *et al.* Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992; 53: 407-414.

12. Gores PF, Najarian JS, Stephanian E, *et al.* Insulin independence in type 1 diabetes after transplantation of purified islets from single donor with 15-deoxyspergualin. Lancet 1993; 341: 19-21.

13. Alejandro R, Lehmann R, Ricordi C, *et al.* Long-term function (6 years) of islet allografts in type 1 diabetes. Diabetes 1997; 46: 1983-1989.

14. Ricordi C, Socci C, Davalli C, *et al.* Isolation of the elusive pig islet. Surgery 1989; 107: 688-694.

15. Korsgren O, Jansson L, Eizirik D, Andersson A. Functional and morphological differentiation of fetal porcine islet-like clusters after transplantation into nude mice. Diabetologia 1991; 34: 379-386.

16. Lui X, Federlin KF, Bretzel RG, *et al.* Persistent reversal of diabetes by transplantation of fetal pig proislets into nude mice. Diabetes 1991; 40: 858-866.

17. Groth CG, Korsgren O, Tibell A, *et al.* Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344: 1402-1404.

18. Davalli AM, Ogawa Y, Scalia L, *et al.* Function, mass and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes 1995; 44: 104-111.

19. Korbutt GS, Elliott JF, Ao Z, *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest 1996; 97: 2119-2129.

20. Marchetti P, Giannarelli R, Cosmi S, *et al.* Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. Diabetes 1995; 44: 375-381.

21. Wright JR, Polvi S, Maclean S. Experimental transplantation with principal islets of teleost fish (Brockman Bodies). Long-term function of tilapia islet tissue in diabetic nude mice. Diabetes 1992; 41: 1528-1532.

22. Ferber S, Beltrandelrio H, Johnson JH, *et al.* Glut-2 gene transfer into insulinoma cell confers both low and high affinity glucose-stimulated insulin release. J Biol Chem 1994; 269: 11523-11529.

23. Knaack D, Fiore DM, Surana M, *et al.* Clonal insulinoma cell line that stably maintains correct glucose responsiveness. Diabetes 1994; 43: 1413-1417.

24. Efrat S, Fusco-Demane D, Lemberg H, *et al.* Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. Proc Natl Acad Sci USA 1995; 92: 3576-3580.

25. Kover K, Moore WV. Development of a method for isolation of islets from human fetal pancreas. Diabetes 1989; 38: 917-924.

26. Hayek A, Beattie GM, Cirulli V, *et al.* Growth factor/matrix-induced proliferation of human adult b-cells. Diabetes 1995; 44: 1458-1460.

27. Ricordi C, Finke EH, Lacy PE. A method for the mass isolation of islets from the adult pig pancreas. Diabetes 1986; 35: 649-653.

28. Hesse UJ, Danis J, Meyer G, *et al.* Preparation and transplantation of pancreatic islet tissue in Landrace pigs and the Munich miniature swine troll. Transplant Proc 1990; 22: 793.

29. Finke E, Marchetti P, Falqui L, *et al.* Large scale isolation, function, and transplantation of islets of Langerhans from adult pig pancreas. Transplant Proc 1991; 23: 772-773.

30. Marchetti P, Finke EH, Gerasimidi-Vazeou A, *et al.* Automated large-scale isolation, *in vitro* function and xenotransplantation of porcine islets on Langerhans. Transplantation 1991; 52: 209-213.

31. Brandhorst D, Hering BJ, Brandhorst H, *et al.* Dietary treatment with soybean oil improves porcine islet culture and reduces islet immunogenecity. Transplant Proc 1994; 26: 613.

32. Basta G, Osticioli L, Tortoioli C, *et al.* Method for mass separation and morphologic qualification of adult porcine pancreatic islets. Xenotransplantation 1995; 2: 120-122.

33. Warnock GL, Katyal D, Okamura J, *et al.* Studies of the isolation, viability, and preservation of purified islets after surgical pancreatectomy in large pigs. Xenotransplantation 1995; 2: 161-164.

34. Sun Y, Zhou D, Vacek I, Sun AM. Isolation of porcine pancreatic islets for xenotransplantation studies: Effects of low collagenase digestion temperatures. Xenotransplantation 1997; 4: 56-61.

35. Schaapherder AFM, Wolvekamp MCJ, Te Bulte MTJW, *et al.* Porcine islets of Langerhans are destroyed by human complement and not by antibody-dependent cell-mediated mechanisms. Transplantation 1996; 62: 29-33.

36. Miyagawa S, Hirose H, Skirakura Y, *et al.* The mechanism of discordant xenograft rejection. Transplantation 1995; 46: 825-830.

37. Johnston PS, Wang MW, Lim SML, *et al.* Discordant xenograft rejection in an antibody-free model. Transplantation 1992; 54: 573-576.

38. McKenzie IFC, Xing P-X, Vaughan HA, *et al.* Distribution of the major xenoantigen (galα(1,3)gal) for pig to human xenografts. Transpl Immunol 1994; 2: 81-86.

39. McKenzie IFC, Koulmanda M, Mandel TE, *et al.* Pig-to-human xenotransplantation: The expression of Galα(1,3)Gal epitopes on pig islet cells. Xenotransplantation 1995; 2: 1-7.

40. Knospe S, Kohler E, Kloting I. Cell-mediated immune reactions against islets of Langerhans in diabetes-prone BB rats. Exp Clin Endocrinol 1987; 89: 290-296.

41. Stock PG, Ascher NL, Platt JL, *et al.* Effect of immunodepletion of MHC class-IIpositive cells from pancreatic islets on generation of cytotoxic T-lymphocytes in mixed islet-lymphocyte coculture. Diabetes 1989; 38 (Suppl 1): 157-160.

42. Nagata M, Yokono K, Hayakawa M, *et al.* Destruction of pancreatic islet cells by cytotoxic T lymphocytes in nonobese diabetic mice. J Immunol 1989; 143: 1155-1162.

43. Nakamura N, Woda BA, Tafuri A, *et al.* Intrinsic cytotoxicity of natural killer cells to pancreatic islets *in vitro*. Diabetes 1990; 39: 836-843.

44. Hayakawa M, Yokono K, Nagata M, *et al.* Morphological analysis of selective destruction of pancreatic β-cells by cytotoxic T lymphocytes in NOD mice. Diabetes 1991; 40: 1210-1217.

45. Nagata M, Santamaria P, Kawamura T, *et al.* Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta-cells in nonobese diabetic mice. J Immunol 1994; 152: 2042-2050.

46. Yoneda R, Yokono K Nagata M, *et al.* CD8 cytotoxic T-cell clone rapidly transfers autoimmune diabetes in very young NOD and MHC class I-compatible scid mice. Diabetologia 1997; 40: 1044-1052.

47. Wolf LA, Coulombe M, Gill RG. Donor antigen-presenting cell-independent rejection of islet xenografts. Transplantation 1995; 60: 1164-1170.

48. Gill RG, Wolf L, Coulombe M. CD4+ T cells are both necessary and sufficient for islet xenograft rejection. Transplant Proc 1994; 26: 1203.

49. Smith RM, Mandel TE. Transplantation treatment for diabetes. Immunol Today 1998; 19: 444-447.

50. Heneine WA, Tibell A, Switzer WM, *et al.* No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. Lancet 1998; 352: 695-699.

51. Leiber MM, Sherr CJ, Benvensiste RE, Todaro GJ. Biologic and immunologic properties of porcine type C viruses. Virology 1975; 66: 616-619.

52. Suzuka I, Shimizu N, Sekiguchi K, *et al.* Molecular cloning of unintegrated closed circular DNA of porcine retrovirus. FEBA 1986; 198: 339-343.

53. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. Nat Med 1997; 3: 282-286.

54. Le Tessier P, Stoye JP, Yasuhiro Y, *et al*. Two sets of human-tropic pig retrovirus. Nature 1997; 389: 681-682.

55. Wilson CA, Wong S, Muller J, *et al.* Type C retrovirus released from porcine primary peripheral blood mononuclear cells infects human cells. J Virol 1998; 72: 3082-3087.

56. Akiyosi DE, Denaro M, Zhu H, *et al.* Identification of a full length cDNA for an endogenous retrovirus of miniature swine. J Virol 1998; 72: 4503-4507.

57. Coffin JM. Retroviridae and their replication. In: Fields BN, Knipe DM, Chanock RM, *et al.*, eds. Fields virology, 2nd edn. New York: Raven, 1990: 1437-1500.

58. Patience C, Patton GS, Takeuchi Y, *et al.* No evidence of pig DNA or retroviral infection in patients with short-term extracorporeal connection to pig kidneys. Lancet 1998; 352: 699-701.

APPENDIX

DETERMINATION OF XENOREACTIVE ANTIBODIES IN SERUM OF YOUNG RABBIT

A-1) OBJECTIVE

To determine whether serum from 3-4 weeks old rabbit contain antibodies against neonatal porcine islet (NPI) cells.

A-2) RATIONALE

It is well-documented that rejection following transplantation between widely divergent (discordant) species (for example, pig-to-human, sheep-to human) is followed by rapid vascular (hyperacute) rejection mediated by preformed natural antibodies and complement (1, 2). These natural antibodies in human serum are directed against pig tissues and binding of these antibodies to the antigen on pig tissues results in the initiation of the complement cascade which eventually destroy the pig tissue within minutes.

The detection of rabbit anti-porcine antibodies in rabbit serum is critical to the studies performed in Chapter II. It is important that the rabbit serum we used for the cytotoxicity assay contain only rabbit complement to eliminate the possibility of rabbit antibodies contributing to the lysis of NPI cells. If rabbit antibodies are present in our experimental system these antibodies to NPI cells can initiate activation of the rabbit complement cascade thus, we will not be able to assess whether human anti-porcine antibodies contribute to the killing of NPI cells.

A-3) EXPERIMENTAL PROTOCOL

Single NPI cell suspension (1×10^6) was incubated with heat inactivated (HI)-rabbit serum from 3-4 weeks old rabbit (1:5 dilution; Pel-Freeze, Brown Deer, WI) for 1 hour on ice. Positive controls for this experiment included NPI incubated in HI-normal adult rabbit serum using the same protocol. NPI cells were then washed and stained with either R-phycoerythrin-conjugated goat anti-rabbit IgG (1:100 dilution; Vector Laboratories, Burlingame, CA) anti-mouse anti-rabbit IgM (0.44 mg/ml; Serotec Canada, Mississauga, ON) for 30 minutes on ice. Rabbit IgM antibody binding on NPI cells was detected by further incubating the cells with CyTM3-conjugated goat anti-mouse antibody (1:100 dilution; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 minutes on ice. Stained cells were washed and analysed by flow cytometry.

A-4) RESULTS

When NPI cells were treated with adult rabbit serum, >90% of the cells bound both rabbit IgG and IgM (Figure A-1). In contrast, NPI cells incubated in young rabbit serum exhibited <1% binding of rabbit IgG or IgM (Figure A-1).

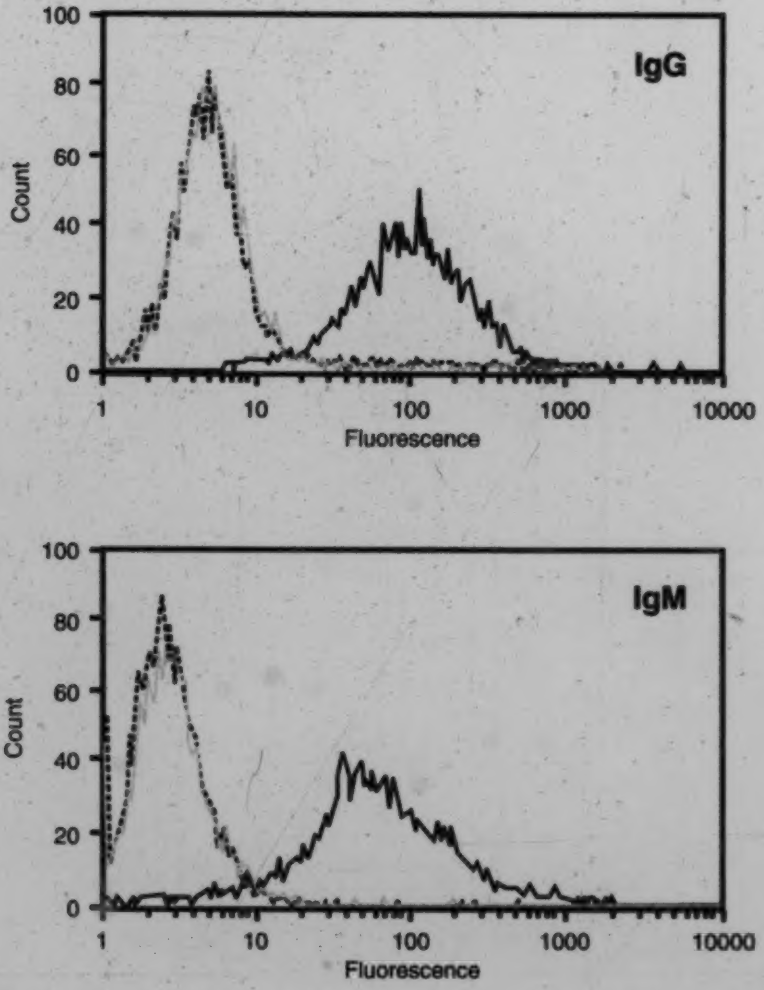

Figure A-1. Binding of rabbit anti-porcine antibodies on NPI cells treated with adult rabbit serum (black solid line), young rabbit serum (gray solid line), or secondary antibody (broken line) as control.

A-5) CONCLUSION

These results show that serum obtained from 3-4 weeks old rabbits does not contain anti-porcine antibodies. This indicates that human and not rabbit antibodies contribute to lysis of NPI cells presented in Chapter II.

A-6) REFERENCES

1. Cooper DKC, Ye Y, Rolf LL Jr, Zuhdi N. The pig as potential organ donor for man. In: Cooper DKC, Kemp E, Reemtsma K, White DJG, eds. Xenotransplantation: The transplantation of organs and tissues between species, 1st edn. New York: Springer-Verlag, 1991: 481-499.

2. Cooley DA, Hallman GL, Bloodwell RD, *et al*. Human heart transplantation: Experience with 12 cases. Am J Cardiol 1968; 22: 804-807.